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Pharmacogenetic studies in osteosarcoma and breast cancer to identify genetic variants involved in treatment efficacy and toxicities

Doctoral Thesis

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La Dra. Anna González Neira, jefe de la Unidad de Genotipado Humano del Centro Nacional de Investigaciones Oncológicas,

CERTIFICA que Doña Daniela Caronia ha realizado bajo su dirección desde el año 2007 hasta la fecha la Tesis Doctoral que lleva por título"*Pharmacogenetic studies in osteosarcoma and breast cancer to identify genetic variants involved in treatment efficacy and toxicities*".

Esta Tesis Doctoral ha supuesto un avance importante en el entendimiento del papel de la variación genética en las diferencias interindividuales encontradas en la respuesta terapéutica en cáncer de mama y osteosarcoma.

Durante estos años, Doña Daniela Caronia ha desarrollado con gran responsabilidad y entusiasmo su labor investigadora en el centro. Su incansable búsqueda de conocimiento, perseverancia y tenacidad ha hecho posible la publicación hasta la fecha de tres trabajos de investigación en revistas internacionales y la presentación de sus resultados científicos en numerosos congresos tanto nacionales como internacionales.

Tras revisar el presente trabajo, considera que reúne la suficiente calidad para su presentación y defensa.

En Madrid, a 28 de Septiembre de 2011

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"…"Te advierto, quien quiera que fueres, Oh! Tú que deseas sondear los arcanos de la Naturaleza, que si no hallas dentro de ti mismo aquello que buscas, tampoco podrás hallarlo fuera. Si tú ignoras las excelencias de tu propia casa, ¿cómo pretendes encontrar otras excelencias? En ti se halla oculto el Tesoro de los tesoros. ¡Oh! Hombre, conócete a ti mismo y conocerás al Universo y a los Dioses."…"

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INDEX

ABBREVIATIONS

5-FU	5-fluouracil
ABC	ATP-binding cassette proteins
ABCA3	ATP-binding cassette, sub-family A (ABC1), member 3
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3
ABCC4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4
ABCC6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2
ADH	Alcohol dehydrogenase
ADRs	Adverse drug reactions
ALDH	Aldehyde dehydrogenase
ASE	Allele-Specific expression
ASO	Allele-Specific Oligos
ASPE	Allele-specific primer extension
AUC	Area under the curve
BLMH	Bleomycine hydrolase
CDD	Cytidine deaminase
CI	Confidence interval
CNV	Copy number variation
COX	Cyclooxigenase
CYP450	cytochrome P450 family
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1
CYP2B6	Cytochrome P450, family 2, subfamily B, polypeptide 6
CYP2C8	Cytochrome P450, family 2, subfamily C, polypeptide 8
CYP2C9	Cytochrome P450, family 2, subfamily C, polypeptide 9
CYP2C19	Cytochrome P450, family 2, subfamily C, polypeptide 19
CYP2D6	Cytochrome P450, family 2, subfamily D, polypeptide 6
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
CYP3A5	Cytochrome P450, family 3, subfamily A, polypeptide 5
DPD	Dihydropyrimidine dehydrogenase
EFS	Event-free survival
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
ERCC2	Excision repair cross-complementing rodent repair deficiency, complementation group 2
ERCC4	Excision repair cross-complementing rodent repair deficiency, complementation group 4
ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5
FAC	5-Fluorouracil, Doxorubicin, and Cyclophosphamide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione S-tranferases
GSTM1	Glutathione S-transferase mu 1
GSTP1	glutathione S-transferase pi 1
GSTT1	Glutathione S-transferase theta 1

GWAS	Genome-wide association studies
HFS	Hand-foot syndrome
HR	Hazard ratio
HWE	Hardy-Weinberg equilibrium
LD	Linkage disequilibrium
LSO	Locus Specific Oligo
MAF	Minor Allele Frequency
MAO	Monoamine oxidase
MDRI	Multidrug resistance protein (ABCB1)
MPO	Monoamine oxidase
mRNA	messenger RNA
NAT	N-acetyltransferases
NER	Nucleotide excision repair
NTC	No template control
OR	Odds ratio
OS	Overall survival
PD	Pharmacodynamics
P-gp	P-glycoprotein (ABCB1, MDR1)
PK	Pharmacokinetics
PCR	Pathological complete response
PCR	Polymerase chain reaction
QC	Quality Control
qRT-PCR	Quantitative real time PCR
RCB	Residual cancer burden
RFLP	Restriction fragment length polymorphism
SD	Standard deviation
SLC	Solute-linked carrier organic anion transporter
SLCO6A1	Solute carrier organic anion transporter family, member 6A1
SLC19A1	Solute carrier family 19 (folate transporter), member 1
SLC28	Solute carrier family 28
SLC29	Solute carrier family 29
SLC31A1	Solute carrier family 31 (copper transporters), member 1
SOD1	Superoxide dismutase 1, soluble
SNP	Single nucleotide polymorphisms
SULT	Sulfotransferases
TP	Thymidine Phosphorylase
TPMT	Thiopurine S-methyltransferase
TYMS	Thymidylate synthase
UGT	Uridine diphosphate glucuronosyltransferases
UTRs	Untranslated region
WT	Wild-type
XPA	Xeroderma pigmentosum, complementation group A
XPC	Xeroderma pigmentosum, complementation group C

ABSTRACT

One of the major problems of modern medicine is the large variability in the way patients respond to drug treatment. A major factor responsible for this variability is genetic variation. Pharmacogenetics is the discipline that studies the effect of genetic variants on the efficacy and toxicities of drug treatment. Individualized pharmacotherapy based on genotype will help to increase treatment efficacy, especially for drugs with a narrow therapeutic window, such as anticancer drugs. In this thesis, we focused on the pharmacogenetics of the treatment of two types of cancer: osteosarcoma and breast cancer.

Osteosarcoma is one of the most frequent bone tumours, occurring mainly in young patients. Standard treatment involves neoadjuvant therapy with a combination of cisplatin, doxorubicin and methotrexate before surgical resection of the primary tumour, followed by postoperative chemotherapy including vincristine and cyclophosphamide. Unfortunately, many patients still relapse or suffer adverse events. As a consequence, the identification of predictors of response and adverse events is of major importance in order to optimize treatment for these patients.
We focused our studies on the genetic pathways related to the pharmacodynamics and pharmacokinetics of the drugs used in osteosarcoma patients. Regarding treatment efficacy, we found that variants in the *ERCC2* gene, which is involved in the nucleotide-excision repair pathway (NER), and the two ATP-binding cassette transporters genes *ABCC3* and *ABCB1*, were associated with treatment outcome and survival. With regard to toxicities, we suggest a role of variation in the NER pathway gene *XPC* in the appearance of cisplatin-induced ototoxicity.
Breast cancer is a public health problem, due to both its incidence and its associated mortality. Docetaxel and doxorubicin are widely used in the treatment of breast cancer, although the benefit is limited to a small proportion of patients. We focused our effort on identifying predictors of efficacy for these two drugs, by studying the genetic variation across the genes involved in the metabolism and transport of these two drugs. We identified two ATP-binding cassette transporter genes, *ABCC2* and *ABCG2* that were associated with drug efficacy in patients treated with doxorubicin or docetaxel, respectively.

A therapeutic alternative for anthracycline- and taxane-resistant breast cancer patients is capecitabine, an oral prodrug of 5-fluouracil (5-FU), which has a more convenient method of administration and has demonstrated to be highly active in breast cancer patients. Unfortunately some patients still suffer severe adverse drug reactions. The most relevant dose-limiting adverse effect is hand-foot syndrome (HFS), characterized by redness, tenderness and peeling of the palms and soles. The appearance of this side-effect leads to capecitabine dose reduction or even interruption of the treatment. We explored the

relationship between genetic variation in genes related to the metabolism of capecitabine and the appearance of HFS and found that genetic variation in the promoter of *CDD* was associated with this toxicity and also with differences in gene expression. That is, it appears that this variation affects gene expression, which could in turn influence the activation to 5-FU, and therefore explain at least part of the susceptibility to experience hand-foot syndrome during capecitabine treatment. We also used a genome-wide approach to identify additional genetic variants that predict severe-grade HFS and found a SNP associated with severe HFS that was validated in an independent population.

The studies performed as part of this thesis provide clear evidence that genetic variation could be used as a predictor of drug efficacy and adverse drug reactions in the treatment of osteosarcoma and breast cancer and could therefore be informative in the design of individualized therapy.

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RESUMEN

Uno de los problemas más relevantes de la medicina moderna es la enorme variabilidad que se observa en relación a la respuesta farmacológica siendo uno de los factores más importantes responsable de esta variabilidad la variación genética.

La farmacogenética es la disciplina que estudia el efecto de las variantes genéticas en la eficacia y toxicidades asociadas a tratamiento con fármacos. La farmacoterapia individualizada basada en el genotipo ayudará a incrementar la eficacia de la terapia y optimizar el tratamiento, especialmente en fármacos que presentan un índice terapéutico muy estrecho como son los fármacos en terapia oncológica.

En esta tesis doctoral nos hemos centrado en la farmacogenética del tratamiento del cáncer, concretamente en osteosarcoma y cáncer de mama.

El osteosarcoma es uno de los tumoures de hueso más frecuente que aparece especialmente en pacientes jóvenes. El tratamiento estándar de este tipo de tumour incluye la terapia neoadyuvante con cisplatino, doxorubicina y metotrexato antes de la resección del tumour primario, seguida de quimioterapia postoperatoria donde se utiliza también vincristina y ciclofosfamida. Desafortunadamente, muchos pacientes recaen o padecen importantes efectos adversos. Resulta por lo tanto de vital importancia para mejorar el éxito del tratamiento de estos pacientes la identificación de predictores de respuesta y de efectos adversos.

Con este objetivo, hemos llevado a cabo el análisis farmacogenético en el conjunto de genes relacionados con la farmacodinámica y farmacocinética de los fármacos utilizados en el tratamiento de estos pacientes y hemos identificado que las variaciones genéticas en el gen *ERCC2*, dentro de la ruta de reparación del ADN de escisión de nucleótido (NER), y los transportadores ABC, *ABCC3 y ABCB1*, están asociadas con la respuesta a tratamiento y la supervivencia de estos pacientes.

El cáncer de mama es un importante problema para la salud pública, dada su incidencia y mortalidad. Docetaxel y doxorubicina son dos fármacos ampliamente utilizados en el tratamiento de este tipo de cáncer, aunque sus beneficios están limitados a un pequeño porcentaje de pacientes. Por esta razón, nos hemos centrado en la identificación de predictores de respuesta para estos dos fármacos y hemos estudiado los polimorfismos en los genes implicados en sus rutas de transporte y metabolismo. Hemos identificado dos transportadores ABC, *ABCC2* y *ABCG2*, que parecen estar relacionados con la eficacia de doxorubicina y docetaxel respectivamente.

Una alternativa terapéutica para aquellos tumoures resistentes a la terapia con antraciclinas o taxanos es la capecitabina, prodroga oral de 5-fluouracilo (5-FU), que presenta una administración más cómoda para el paciente y que ha demostrado ser muy activa en este tipo de tumoures. Lamentablemente, algunos de los pacientes sufren severos efectos adversos. El más limitante es el denominado síndrome de mano-pié (SMP), que se caracteriza por la aparición de enrojecimiento, sensibilidad y descamación de las palmas de las manos y las plantas de los pies. La aparición de este efecto adverso conlleva desde la reducción de la dosis de fármaco al paciente hasta a la interrupción del tratamiento. Hemos explorado la relación existente entre esta toxicidad y los genes relacionados con el metabolismo de este fármaco identificando una variante genética en el promotor del gen *CDD* que está implicado en la conversión de capecitabina a 5-FU, asociada con la aparición del SMP y que altera su expresión génica. Este efecto en la expresión parece influir en la activación del fármaco a 5-FU explicando en parte la susceptibilidad observada a sufrir SMP. Además con el objetivo de identificar nuevas variantes asociadas con este síndrome, hemos llevado a cabo el análisis de la variación del genoma completo identificando un nuevo polimorfismo asociado a la aparición del SMP y que ha sido replicado en una serie de pacientes independiente.

Todos los resultados obtenidos en esta tesis doctoral suponen una clara evidencia del importante papel que juega la variación genética en las diferencias interindividuales observadas tanto en la eficacia como en toxicidad en el tratamiento de osteosarcoma y cáncer de mama y que por lo tanto podrían ser aplicados en el diseño de una medicina individualizada en estos pacientes.

INTRODUCTION

1. Pharmacogenetics

Interindividual variation in the sensitivity to drugs represents a significant therapeutic obstacle for clinicians. This variation could affect the effectiveness of a treatment by impairing drug response or inducing adverse drug reactions (ADRs).

The percentage of non-responders to treatment among patients with the most common diseases ranges from 20% to 75%, with the highest rates observed in oncology (Spear *et al*, 2001). On the other hand, the appearance of adverse drug reactions limits the effectiveness of a therapy, since severe cases require treatment discontinuation or dose reduction. Adverse drug reactions account for nearly 7% of all hospital admissions in Western countries (Budnitz *et al*, 2006), (Gurwitz & Motulsky, 2007), (Pirmohamed *et al*, 2004).

For a selected treatment, the effect observed in a group of patients could range from: a) response without toxicities, b) response to therapy but development of toxicities, c) non-response without toxicities, and d) non-response to therapy and development of toxicities (Figure 1).

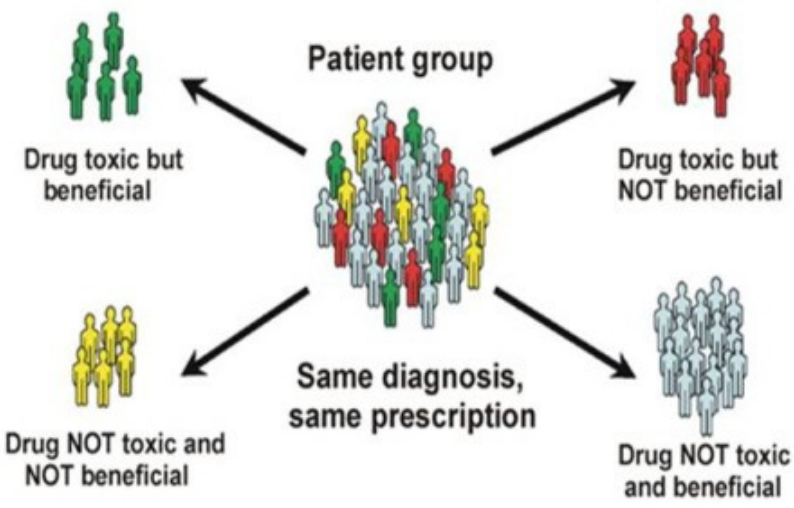


Figure 1. Interindividual variability of drug effects. Patients can be divided into four groups according to drug effects: responders without toxicity (in grey), responders with toxicity (in green), non-responders without toxicity (in yellow), and non-responders with toxicity (in red).

Although multiple factors can influence this interindividual heterogeneity, such as age, organ function, drug interactions and environmental factors, genetic factors play a major role. It is estimated that genetics account for between 20 and 95 percent of the variability in drug disposition and effects.

The impact of inherited genetic variability on drug response and therapy-induced toxicity has been studied intensively in the past decades. This has been a rapidly growing field of research since the release of the first draft of the human genome sequence, and is known as pharmacogenetics (Gibson *et al*, 2007), (Nebert, 1982), (Sjoqvist, 1999).

The potential of pharmacogenetics lies in the identification of the right drug at the right dose for each patient. Pharmacogenetic approaches aim to identify patients unlikely to respond, or likely to develop severe toxicity, to a drug before treatment decisions are made. This approach should ultimately facilitate the development individualized therapy through tailored dosing or treatment modification strategies, thereby avoiding genetically altered drug pathways.

1.1. Human genetic variation

It is estimated that the average difference in nucleotide sequence between a randomly chosen pair of humans is approximately 0.1% (Shastry, 2009). A genetic polymorphism is defined as a locus at which the least frequent allele is present in more than one percent of the population; less common variants are referred to as mutations (Nebert, 2000b). The most common genetic variant is the single nucleotide polymorphism (SNP). This type of variant can occur anywhere in the genome, within the coding sequences of genes, in non-coding regions of genes, or in intergenic regions. SNPs in the coding region are called non-synonymous when they lead to an amino acid substitution and synonymous SNPs when they do not. SNPs can also alter promoter activity, mRNA conformation and stability, or they can affect gene splicing, transcription factor binding, or the sequence of non-coding RNA. Other forms of common genetic variation are short tandem repeats (STRs), also known as microsatellites, variable number tandem repeats (VNTRs) and small (<1 Kb) insertion and deletion (In-del) polymorphisms (Feuk *et al*, 2006), (Mills *et al*, 2006). Technological developments in recent years have enabled the study of another type of polymorphic variation that seems to involve 0.78 % of the human genome sequence (Conrad *et al*, 2009). This consists of gains or losses of a region larger than one kilobase which are referred to as copy number variants (CNVs) (Zhang *et al*, 2006). While in general CNVs leave the primary amino acid sequence unchanged, the presence of multiple gene copies can lead to a general increase in the corresponding protein activity. Conversely, entire gene deletions may occur, leading to the absence of protein activity.

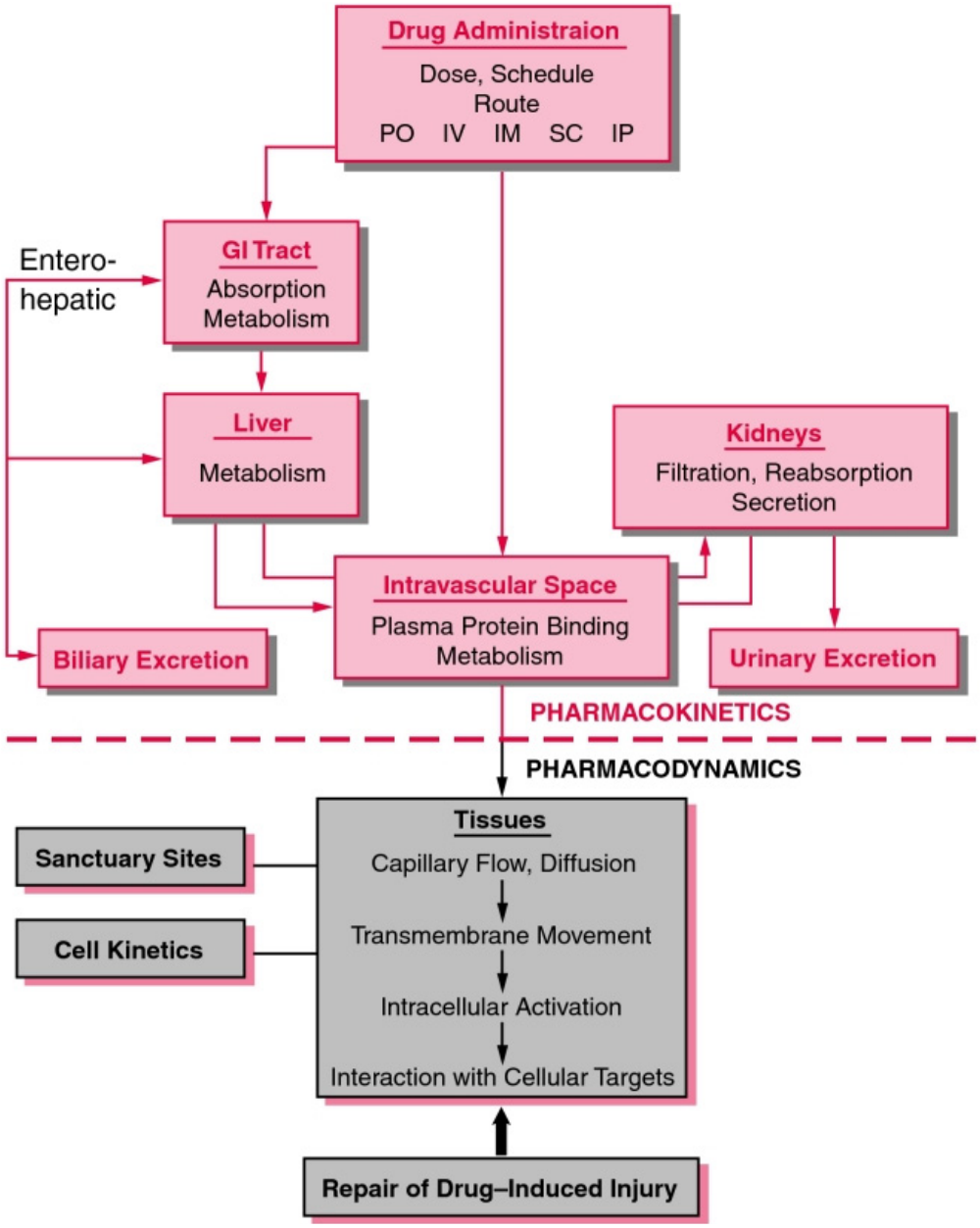
There are a multitude of publications reporting associations between SNPs and susceptibility to many complex traits and common diseases such as diabetes (Yasuda *et al*, 2008), coronary

artery disease (Willer *et al*, 2008) and cancer (Antoniou *et al*, 2008). More recently, CNVs have also been found to be associated with susceptibility to human diseases (McCarroll & Altshuler, 2007), (Tam *et al*, 2009), (Wain *et al*, 2009), (Lesch *et al*, 2010), (Bae *et al*, 2010). In addition, in the pharmacogenetic field there are ample published examples of causal relationships between SNPs and CNVs and drug response, which account for clinically importance differences in individual outcomes from therapy (Goetz *et al*, 2007), (Cheok & Evans, 2006), (Laverdiere *et al*, 2002), (Kweekel *et al*, 2008), (Mallal *et al*, 2002), (Kirchheiner *et al*, 2001), (Hulot *et al*, 2006).

1.2. Germline genetic variation and its relevance in pharmacokinetics and pharmacodynamics

Genetic polymorphisms may affect the pharmacokinetic (PK) and pharmacodynamic (PD) behavior of a drug, observed as differences in drug transport, metabolism and pharmacodynamic effects (Figure 2).

Pharmacogenetic studies have shown that germline polymorphisms in genes related to drug transport, phase I and II metabolizing enzymes, drug targets and other genes related to drug response, can have a major effect on the PK and PD of drugs (Zhou *et al*, 2008).



(From Kufe DW, Pollock RE, Weichselbaum RR, et al. Holland-Frei Cancer Medicine. 6th edition.)

Figure 2. Pharmacokinetics and Pharmacodynamics of drugs. Pharmacokinetics control drug absorption, distribution, metabolism and elimination, while pharmacodynamics encompasses the interaction of a drug with its targets and repair mechanisms. *PO: per os, IV: intravenous, IM: intramuscular, SC: subcutaneous, IP: intraperitoneal.*

1.2.1 Pharmacokinetics

The genes involved in pharmacokinetics are those that control drug absorption, distribution, metabolism, and excretion. Most drug metabolizers and transporters contain many genetic polymorphisms, which might account for large interindividual variability in the plasma concentration of drugs.

A) Transporters

Two types of transport superfamilies, ATP-binding cassette (ABC) (Sissung *et al*, 2008c) efflux pumps and solute-linked carrier (SLC) influx proteins (Kindla *et al*, 2009) are responsible for the majority of drug transport (Zhou *et al*, 2008). The ABC transporters are heavily involved in the absorption and disposition of many clinically used drugs, including anticancer drugs. The most widely studied ABC transporter is ABCB1 (P-glycoprotein, P-gp), which transports many anticancer drugs such as doxorubicin, vincristine, vinblastine, etoposide, irinotecan, paclitaxel and docetaxel (Schwab *et al*, 2003) and is a major contributor to drug resistance in cancer treatment (Huang *et al*, 2004). Other ABC transporters such as ABCC1, ABCC2 and ABCG2 have been related to the transport of the anti-cancer drugs doxorubicin, etoposide, methotrexate, vincristine and cisplatin, among others (Huang, 2007), (Cascorbi & Haenisch, 2009). Of the SLC transporters, the reduced folate carrier (RFC) family (SLC19), the amino acid transporters (SLC28) and the CTR copper transporter family (SLC31A) all mediate the uptake of, and chemosensitivity to, anticancer drugs (Huang & Sadee, 2006) .

B) Phase I Metabolizing Enzymes

Phase I metabolizing enzymes mediate drug oxidation, reduction and hydrolysis, which leads to activation or inactivation of the drug. The oxidation reactions involve the cytochrome P450 (CYP450) family (Redlich *et al*, 2008), as well as monoamine oxidase (MAO), cyclooxigenase (COX), alcohol dehydrogenase (ADH), and aldehyde dehydrogenase (ALDH). The CYP450 genes are the most important group of phase I metabolizing enzymes (Eichelbaum *et al*, 2006); the members of this family that are especially involved in chemotherapeutic metabolism are *CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4 and CYP3A5* (Rodriguez-Antona & Ingelman-Sundberg, 2006), (van Schaik, 2005), (Bosch *et al*, 2006). Other Phase I enzymes that metabolize anticancer agents are the reduction enzyme dihydropyrimidine dehydrogenase (DPD) (Diasio & Harris, 1989), (Wei *et al*, 1996) which detoxifies fluoropyrimidines, the deaminating enzyme cytidine deaminase (CDD) which metabolizes gemcitabine, capecitabine and cytarabine (Gilbert *et al*, 2006), and the hydrolyzing enzyme bleomycine hydrolase (BLMH) which metabolizes bleomycin(Koldamova *et al*, 1998).

C) Phase II Metabolizing Enzymes

Phase II enzymes polarize lipophilic drugs through conjugation with a polar substrate which facilitates renal and biliary excretion. The phase II conjugation reactions are catalyzed by glutathione-S-transferases (GSTs), uridine diphosphoglucuronosyl transferases (UGTs),

methyltranseferase such as thiopurine S-methyltransferase (TPMT), sulfotransferases (SULTs), and N-acetyltransferases (NATs). GSTs catalyzes the conjugation of glutathione to both endogenous substrates and xenobiotics, including anticancer drugs such as platinum agents, cyclophosphamide and anthracyclines (Salinas & Wong, 1999), (Hayes & Pulford, 1995). Copy-number variants (CNVs) resulting in diminished or missing enzyme activity have been described in the *GSTT1* and *GSTM1* genes (Bruhn *et al*, 1998), (Bernardini *et al*, 2002). These CNVs have been reported to be associated with tumour response in the treatment of different types of cancer (Cotton *et al*, 2000), (Ambrosone *et al*, 2001). UGT catalyzes the glucuronidation of bilirubin and drugs such irinotecan (UGT1A1, UGT1A7 and UGT1A9) (Rosner *et al*, 2008), (Villeneuve *et al*, 2003) and tamoxifen (UGT2B15) (Wegman *et al*, 2007). TPMT catalyzes the S-methylation of 6-Mercaptopurine (6-MP) and azathioprine (Lennard, 1992), drugs that are used in the treatment of leukemias and lymphomas (Sahasranaman *et al*, 2008). A small number of anticancer drugs are substrates for SULTs (tamoxifen) (Falany *et al*, 2006) or NATs (amonafide) (Innocenti *et al*, 2001).

1.2.2 Pharmacodynamics

The area of pharmacodynamics explores the effects of drugs on receptors and other response mechanisms. Interindividual differences in the expression and activity of proteins involved in drug effects may explain part of the observed population heterogeneity in response to treatment.

Genes involved in the pharmacodynamics of chemotherapeutic agents include thymidylate synthase (*TYMS*) and the DNA repair genes.

Anticancer drugs targeting TYMS include, among others, fluoropyrimidines: the main active metabolite of these drugs inhibits TYMS leading to DNA synthesis arrest followed by cell death (Grem, 2000), (Rustum *et al*, 1997), (Welsh *et al*, 2000). The expression levels of TYMS have been related to fluoropyrimidine sensitivity (Leichman *et al*, 1997), (Nishimura *et al*, 1999). Furthermore, a tandem repeat polymorphism in the 5' promoter as well as a 6 bp deletion in the 3'UTR (Mandola *et al*, 2004) (Ulrich *et al*, 2000) have been found to be associated with *TYMS* gene expression (Mandola *et al*, 2003) (Kawakami *et al*, 1999).

Various genetic pathways are involved in DNA damage repair after exposure to DNA-damaging agents (Hoeijmakers, 2001); the nucleotide excision repair (NER) pathway one of these and is implicated in the removal of platinum induced DNA adducts (Zamble *et al*, 1996) (Figure 3).

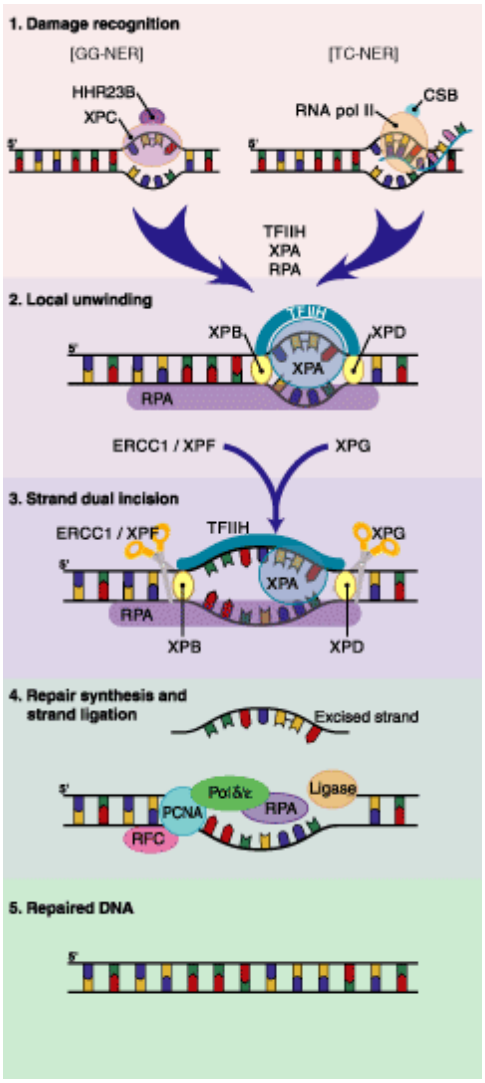


Figure 3. NER pathway. The NER pathway is formed by a complex network of many proteins involved in lesion recognition, excision, DNA resynthesis and ligation.

Both SNPs in, and alterations in the expression of, NER genes are associated with cisplatin resistance (Dabholkar *et al*, 2000) (Quintela-Fandino *et al*, 2006). SNPs in the *ERCC1* and *ERCC2* (Park *et al*, 2001) genes have been found to be associated with platinum response in different clinical studies. In particular, the *ERCC2* Lys751Gln variant was associated with reduced survival and worse prognosis in platinum-treated non-small-cell lung cancer patients (Gurubhagavatula *et al*, 2004) and colorectal cancer patients (Ruzzo *et al*, 2008), respectively.

1.3. Pharmacogenetic strategies

There are essentially two strategies that can be adopted to discover pharmacogenetic markers: candidate gene approaches and the genome-wide approach (GWAS).

Candidate gene approaches utilize knowledge of the pathways involved in the pharmacokinetics and pharmacodynamics of therapeutic agents to identify relevant polymorphisms in established or suspected drug-related genes. These studies are also termed hypothesis-driven association studies. Most of the pharmacogenetic studies performed to date have been based this approach. However, in many cases, one single genetic variant does not sufficiently explain the wide interindividual variability in drug response. Differences in response to drugs often likely due to polygenic effects, such that combinations multiple polymorphisms in one or more genes within and across many pathways explain the full genetic predictive value for clinical outcome. Furthermore, it is possible that not only genes encoding metabolic enzymes are relevant, but also genes related to a wide range of processes, such as immune response (Daly, 2010), (Wu *et al*, 2011), (Yang *et al*, 2009). Other, as yet poorly understood, or unknown, processes may also be involved.

To more fully understand the genetic basis of drug response and adverse drug reactions, genome wide approaches (GWAS) devoid of a priori assumption have begun to be adopted to gain a further knowledge of the genetic basis of therapy response. These studies analyse from 500 thousand to 5 million SNPs across the entire genome. They are not hypothesis driven and are therefore able to identify associations with common variants in genes that were previously not known to be relevant. The identification of such genes could reveal novel mechanisms involved in drug response.

These arrays are composed mainly of tagSNPs, based on the data available from the Hapmap Project, which ensures coverage of common variation across the entire genome; that is, genotyping these polymorphisms provides information on additional polymorphisms in linkage disequilibrium with them. Due to the very large number of SNPs interrogated, it is essential to allow for the possibility of obtaining false positive results, but this limitation can be addressed by performing replication studies in independent series of patients.

Another limitation of GWAS is that in many cases the biological meaning of associations identified is difficult to assess, because most of the SNPs interrogated are located outside, or in the intronic regions, of genes. Because of this, the post-GWAS era of this scientific research involves the task of identify the causal variant responsible for the observed association, and this is resulting much more difficult than expected.

In recent years, the number of GWAS studies in pharmacogenetics has increased. In some cases, such as warfarin dose requirement (Cooper *et al*, 2008), (Takeuchi *et al*, 2009) and response to clopidogrel (Shuldiner *et al*, 2009) these have confirmed previous findings from

candidate gene studies. In other cases, the GWAS approach has identified novel pharmacogenetic associations that would be unlikely to have been detected by candidate pathway studies; examples include HLA genotypes and susceptibility to flucloxacillin-induced liver injury (Daly *et al*, 2009), *SLCO1B1* and methotrexate pharmacokinetics (Trevino *et al*, 2009), and *TCL1A* and musculoskeletal adverse events induced by aromate inhibitors (Ingle *et al*, 2010). Until now, only genetic variants with large effects have been detected, with OR higher than 4 (Daly, 2010); this is at least in part due to the difficulty of recruiting large numbers of cases for pharmacogenetic studies. Furthermore, the analysis of data from pharmacogenetic studies is complicated by the way the phenotypes of response and toxicities to treatment are measured in the form of a subjective, ordinal scale (grade). To avoid biases and more efficiently prioritise samples for genotyping, one possible strategy is to focus the analysis on extreme phenotypes, including only patients at the extreme ends of the distributions of the outcomes of interest. (Crowley *et al*, 2009), (Turner *et al*, 2008), (Nebert, 2000a).

2. Cancer and Pharmacogenetics

Interindividual variability in treatment response and toxicity, while observed for most drugs, is of particular concern in oncology because the drugs have a narrow therapeutic range. That is, the difference between the dose causing toxicity and that required for the drug to be effective is relatively small (Leeder, 1998). While inappropriate treatment can give rise to selection for more aggressive and resistant cancer cells, patients are often treated according to standard regimens that do not take into account individual genetic variability; this may also lead to the appearance of resistance during treatment.

Pharmacogenetic studies in clinical oncology typically assess the relationship between genetic polymorphisms and drug-related toxicities, treatment efficacy and survival after treatment. Knowledge of the clinical impact of genetic variants may then enable patient-tailored therapy. In this thesis, we focused our studies on the pharmacogenetics of chemotherapeutic agents used in two types of cancer: osteosarcoma and breast cancer.

2.1. Cancer chemotherapy

The main chemotherapeutic agents can be classified by mechanism of action:

a) Antimetabolites are structural analogs of endogenous metabolites involved in DNA and RNA synthesis. They interfere with nucleic acid synthesis and are therefore active in tumours with a high growth rate. Antimetabolites can be divided into folate

antagonists, such as methotrexate (Bertino *et al*, 1996), and pyrimidine and purine analogs, such as 6-mercaptopurine, 5-fluorouracil, cytosine arabinoside, 5-azacytidine and gemcitabine.

b) Alkylating agents and platinum compounds are a heterogeneous group of compounds that interact with DNA and alkylate nitrogen at the 7 position of guanine, causing DNA cross-links. The most commonly used alkylating agents in cancer therapy are cyclophosphamide, ifosfamide, melphalan and chlorambucil, while the main platinum compounds are cisplatin and carboplatin (Rabik & Dolan, 2007).

c) Anthracyclines are positively charged at physiologic pH and intercalate into double-stranded DNA and produce structural changes that interfere with DNA and RNA synthesis. In addition, they inhibit topoisomerase II, a key enzyme in the synthesis of DNA. This interaction alters the synthesis and transcription of DNA. Daunomycin and doxorubicin are the most important anthracyclines (Gewirtz, 1999).

d) Microtubule-targeting agents target and disrupt the microtubules, components of the cellular cytoskeleton including the mitotic spindle apparatus, leading to metaphase arrest. Microtubule-targeting agents can be divided into vinca alkaloids (vincristine, vinblastine, vinorelbine), which inhibit tubulin assembly, and taxanes (paclitaxel and docetaxel), which promote microtubule assembly and stability, thus blocking the cell cycle in G2/M phase (Karantanis *et al*, 1994).

e) Topoisomerase inhibitors: topoisomerase I inhibitors interrupt the elongation phase of DNA replication and include the camptothecin analogs irinotecan and topotecan (Hsiang *et al*, 1985); topoisomerase II inhibitors stabilize the DNA-topoisomerase complex, leading to inhability to synthesize DNA and cell cycle arrest; the most commonly used is etoposide (Wozniak & Ross, 1983).

2.2. Osteosarcoma

Osteosarcoma is one of the most frequent bone tumours, comprising about 20% of primary bone sarcomas (Marina *et al*, 2004). It is the most frequent malignant bone tumour in children and adolescents (Longhi *et al*, 2006), with a peak in disease incidence in the second decade of life

2.2.1. Osteosarcoma Treatment

Standard treatment of osteosarcoma is based on combinations of different drugs: neoadjuvant chemotherapy with methotrexate, cisplatin and doxorubicin, followed by surgery and post-

operative chemotherapy comprising methotrexate, cisplatin, doxorubicin, cyclophosphamide and vincristine (Longhi *et al*, 2006).

2.2.1.1. Efficacy in osteosarcoma treatment

One of the most consistent prognostic factors is osteosarcoma is histological response to preoperative treatment (Glasser *et al*, 1992), evaluated as the percentage of tumour necrosis, which is associated with event-free-survival (EFS) and overall survival (Bielack *et al*, 2002). The introduction of multiagent chemotherapy has improved the 5-year disease-free survival rate to 60-70% over the past three decades.

Despite chemotherapy and surgery, some (about 30%) patients still relapse or metastasize (Chou & Gorlick, 2006). Since strategies such as the intensification of chemotherapy and the addition of other agents have not led to long-term benefits, research efforts have been directed towards identifying factors that predict drug response and clinical outcome.

2.2.1.2. Toxicities in osteosarcoma treatment

The most frequently reported acute toxicities of osteosarcoma treatment are alopecia, myelosuppression, mucositis, nausea and vomiting, all common complications of most chemotherapeutic treatments. Long-term adverse effects of osteosarcoma treatment include cardiac toxicity, acute and chronic nephrotoxicity, neurotoxicity, hearing loss and infertility (Janeway & Grier).

Specifically, in this thesis we will focus on cisplatin-induced hearing loss, which is observed in approximately 40% of osteosarcoma patients treated with the drug. Cisplatin-induced hearing loss, also referred to as ototoxicity, is usually bilateral and irreversible, and is particularly serious in the children. Loss of hearing at an early age hampers speech, cognitive and social development. Pediatric patients treated with cisplatin at cumulative doses approaching 400 mg/m^2 experienced worsening of their hearing long after treatment; while audiograms showed hearing loss in 5% of patients before the end of therapy, 44% had significant hearing loss after more than two years of follow-up (Bertolini *et al*, 2004). Thus, there is an imperative need for predictive markers for this adverse event.

2.3. Breast Cancer

Breast cancer is the most frequent cancer in women in developed countries (Parkin *et al*, 2005). In the recent decades there have been many improvements in the treatment of breast

cancer. Nevertheless, there is still substantial interindividual variability in drug response and toxicity.

2.3.1. Breast Cancer Treatment

The therapeutic strategy adopted in breast cancer is based on locoregional disease control, (treatment of the affected breast and regional lymph node chains) and treatment of distant disease (micrometastases) in the early stages or metastases in advanced stage. Therefore, the treatment of breast cancer is very often multidisciplinary, including surgery, radiotherapy, chemotherapy and hormone therapy as well as treatments directed at specific molecular targets. In patients with hormone receptor-positive breast cancer, endocrine therapy will be used at some stage during the course of the disease, as long as there are no contraindications. Chemotherapy is indicated in the palliative setting, when the disease is hormone-receptor negative, unresponsive to endocrine treatment or highly aggressive. Prognosis is generally good for patients diagnosed with early-stage breast cancer, with a 5-year disease-free survival rate of 90% and an overall survival rate up to 98% for patients with localized disease (SEER Cancer Statistics reviews(Review, 1975-2008)). However, as many as 30% of women diagnosed with early-stage breast cancer will eventually progress to or relapse with locally advanced or metastatic disease (Brewster *et al*, 2008). Anthracyclines and taxanes are the agents that have shown the highest efficacy in all settings of breast cancer. However, treatment failure due to resistance occurs in >90% of patients with metastatic breast cancer (Longley & Johnston, 2005). Capecitabine is an approved chemotherapeutic option for resistant breast cancer (Blum *et al*, 2001).

Specifically, in this thesis we will focus on doxorubicin (anthracycline) and docetaxel (taxane) in the neoadjuvant setting of locally advanced breast cancer and capecitabine (antimetabolite) in metastatic breast cancer.

2.3.1.1. Docetaxel efficacy and toxicities

Docetaxel is a semisynthetic taxane (microtubule-acting agent) synthesized from extracts of the needles of the European yew tree (*Taxus baccata*) (Karantanis *et al*, 1994). It has been proposed for the neoadjuvant treatment of locally advanced breast cancer (Tham *et al*, 2005), (Gradishar *et al*, 2005), (Martin *et al*), (Stemmler *et al*, 2001). Docetaxel as a single agent produces objective responses in up to 60% of patients with metastatic breast cancer (Dieras *et al*, 1996), (Ravdin *et al*, 1995). The usual method of administration is a 1-hour infusion every 3 weeks, at a dose of 75 to 100 mg/m^2 (Harvey *et al*, 2006). Docetaxel as a single agent has been

reported to produce a higher response rate and longer time to progression than full-dose doxorubicin, although survival was equivalent for the two drugs (Chan, 1997), (Chan *et al*, 1999). Docetaxel in the neoadjuvant setting of locally advanced breast cancer improved disease-free and overall survival compared with the combination of cyclophosphamide, doxorubicin, vincristine and prednisólone (Hutcheon *et al*, 2003). Furthermore, neoadjuvant docetaxel had a similar treatment response rate to that of doxorubicin as monotherapy in a trial that included patients with locally advanced breast cancer (Martin *et al*, 2011). This trial identified ER status and tumour subtype as predictors of response to docetaxel.

The most common adverse effects of docetaxel are hematologic toxicity (70-90% of patients), (70-90%) neurological reactions (4-10%) (Vasey *et al*, 2001), and persistent alopecia (up to 12% of patients) (Martin *et al*, 2005).

2.3.1.2. Doxorubicin efficacy and toxicities

Doxorubicin (anthracycline) is also used in the neoadjuvant treatment of primary breast cancer (Arriola *et al*, 2006), (Martin *et al*, 2011). Thyrty to 50% of metastatic breast cancer patients achieve an objective regression after single-agent anthracycline therapy (Chan *et al*, 1999). Doxorubicin-containing combinations are probably the most commonly used regimens today The 5-fluorouracil, doxorubicin, cyclophosphamide (FAC) regimen came into use in 1973; successively, other doxorubicin-containing combinations were reported (A'Hern *et al*, 1993). Genomic predictors of doxorubicin response were ER status and topo2A expression (Martin *et al*, 2011). However, these factors only explain a part of the variability observed in response to this agent. The limiting toxicities of doxorubicin are mielotoxicity, nausea, and cumulative dose-dependent cardiotoxicity (Amar *et al*, 2009). Cardiotoxicity is a side effect specific to doxorubicin and is observed in 0-4% of patients treated with this drug. It is characterized by congestive heart failure and occurs weeks to months after the completion of therapy (Shan *et al*, 1996).

2.3.1.3. Capecitabine efficacy and toxicities

Capecitabine (Xeloda) is a 5-fluorouracil prodrug. It is almost 100% orally available and peak plasma levels are present within 1.5-2 hours after rapid absorption (Reigner *et al*, 2001). Capecitabine is an efficient agent that, unlike 5-fluorouracil, does not require continuous infusion and therefore offers patients more freedom from hospital visits and avoids the inconvenience and complications associated with infusion devices. This drug is recommended as treatment for metastatic breast cancer in particular and is also used in the treatment of colorectal cancer (McKendrick & Coutsouvelis, 2005).

Capecitabine monotherapy has been shown to have high antitumor activity in anthracycline- and taxane-pretreated metastatic breast cancer, with an objective response rate ranging from of 15 to 29% (Blum *et al*, 2001), (Reichardt *et al*, 2003), (Fumoleau *et al*, 2004), (Wist *et al*, 2004). The combination with docetaxel increased the response rate from 30% for docetaxel as monotherapy to 42% (O'Shaughnessy *et al*, 2002). Capecitabine is currently approved by the US Food and Drug Administration (FDA) for use in breast cancer patients as single agent following resistance to both anthracycline-based and paclitaxel-based regimens or in those in whom further anthracycline treatment is contraindicated. It is also approved in combination with docetaxel after the failure of earlier anthracycline based chemotherapy (Blum *et al*, 2001), (Reichardt *et al*, 2003), (Fumoleau *et al*, 2004), as well as in first-line single-agent therapy for patients with advanced or metastatic colorectal cancer, when single agent fluoropyrimidine therapy is preferred. Capecitabine was more effective than 5-FU in clinical trials (26% efficacy vs 17%) (Hoff *et al*, 2001), (Van Cutsem *et al*, 2001). Capecitabine has also been evaluated as first-line treatment in metastatic colorectal cancer patients as well as in the adjuvant setting, in combination with oxaliplatin (Shields *et al*, 2004), (Cassidy *et al*, 2004), (Zeuli *et al*, 2003).

Although this drug offers a more selective alternative to 5-FU, since it is converted into the active form specifically in the tumour cells (Walko & Lindley, 2005), thereby lowering the adverse effects related to 5-FU (Miwa *et al*, 1998), capecitabine-induced hand-foot syndrome (HFS) occurs in substantial proportion (almost 30%) of treated patients. It is characterized by tenderness, redness and swelling of the palms and soles (Figure 4), and often requires dose reduction or even treatment interruption (Nagore *et al*, 2000).

Figure 4. Hand-foot syndrome. HFS affects the soles of the feet and the palms of the hands causing numbness, erythema, swelling, ulceration, desquamation and severe pain.

Some polymorphisms such as three 28-bp repeats in the 5' UTR of the *TYMS* gene containing a G>C mutation in the second repeat, a 6bp deletion in the 3' region of *TYMS*, and the inactivating mutation IVS14+1G>A in the *DPD* gene (Ulrich *et al*, 2002), (van Kuilenburg, 2004), (Largillier *et al*, 2006) have been related to severe global toxicity appearance when ADRs such as myelosuppression, hematologic toxicity, diarrhea, and HFS are grouped. However, few pharmacogenetic studies have been performed (Gonzalez-Haba *et al*, 2010), (Ribelles *et al*, 2008), (Shahrokni *et al*, 2009) considering exclusively HFS appearance. Therefore, genetic risk factors for HFS remain to be elucidated.

Specifically, in this thesis we will focus on the identification of genetic predictors of the appearance of hand-foot syndrome during treatment with capecitabine.

OBJECTIVES

The principal purpose of this thesis was to identify genetic markers associated with chemotherapeutics efficacy and toxicity. This data could provide the basis for an individualized pharmacotherapy in the treatment of human cancers, in particular in osteosarcoma and breast cancer.

To achieve this, we placed the following specific aims:

1. To identify genetic markers associated with osteosarcoma treatment response and toxicity by:

    1.1. Studying DNA-repair pathway gene variants related to cisplatin-related ototoxicity and response

    1.2. Studying drug metabolism/transport gene variants related to treatment efficacy and survival

2. To identify genetic markers associated with breast cancer treatment response and adverse events by:

    2.1. Studying drug metabolism/transport gene variants related to docetaxel and doxorubicin treatment efficacy

    2.2. Studying genetic variants related to capecitabine- induced hand-foot syndrome by:
        2.2.1. Candidate pathway approach
        2.2.2. Genome-wide (GWAS) approach

MATERIALS & METHODS

1. Biological samples

1.1. Patients and clinical data

The cancer patients studied in this thesis were oncology patients from four different hospitals and one Pharmaceutical company: University Clinic of Navarra in Pamplona, Hospital Clinico San Carlos in Madrid, Hospital Universitario Virgen de la Victoria, Malaga, Spain, Hospital General Universitario Gregorio Marañon and Roche Pharmaceutical Company (www.roche.com). The first hospital participated in the osteosarcoma studies and the rest of the centers in the breast cancer studies. All the studies were approved by The Ethical Committees of the respective hospitals, and informed consent was given by the patients.

1.1.1. Samples from osteosarcoma studies

One hundred and two patients diagnosed with osteosarcoma at the University Clinic of Navarra, Pamplona, Spain, between 1986 and 2009 were enrolled. All samples were obtained with written informed consent from patients, their parents, or both. Ethical approval of the study was granted by the Ethics Committee of the University Clinic.

Patients were treated preoperatively with intravenous (i.v.) doxorubicin (3 courses at 25-30 mg/m^2/day for 3 days), i.v. methotrexate (4 courses of up to 14 g/m^2/day for 1 day) and intra-arterial cisplatin (3 courses at 35 mg/m^2/day for 3 days). After surgery, the adjuvant chemotherapy included methotrexate (10 g/m^2/day for 1 day and folinic acid rescue) and alternate cycles of i.v. cisplatin/doxorubicin or i.v. actinomycin D (0.45 mg/m^2/day for 3 days), cyclophosphamide (500 mg/m^2/day for 3 days), and vincristine (1.5 mg/m^2/day for 1 day) for up to 48 weeks of treatment.

Response to treatment was determined histologically by the percentage of necrosis induced in the tumour after neoadjuvant chemotherapy. Patients with less than 90% necrosis were classified as poor responders and those with 90% necrosis or higher, as good responders (Bacci *et al*, 2003).

Other clinical data including age, sex, tumour location, metastatic events (both at diagnosis and at follow-up) and relapses (disease recurrence in the same bone) were systematically recorded from the clinical records. Only conventional high-grade osteosarcomas were included, regardless of metastatic stage at diagnosis.

Study I: Osteosarcoma and DNA repair genes: treatment efficacy and ototoxicity

For this study analyzing DNA-repair gene variants related to cisplatin-related ototoxicity and reponse, patients whose neoadyuvant regimen did not include cisplatin were excluded. Therefore, a total of 70 were considered in the analysis of response to treatment. The characteristics of the patients are shown in Table 1. The median age at diagnosis was 15 years (range 4 to 34 years). At the time of diagnosis, 16% of the patients already presented metastasis, while 21% developed metastasis during follow-up. At the time of the final analysis on March 2007, the median follow-up was 91 months (range 10 to 272).

The cumulative dose of platinum relative to total body surface was recorded for each patient, considering both intraarterial neoadjuvant and intravenous adjuvant treatment with cisplatin. Cisplatin was given at a dose ranging from 120 to 1131 mg/m^2 for intraarterial treatment and from 83 to 948 mg/m^2 for intravenous treatment.

Event-free-survival (EFS) was considered from tumour diagnosis to the first of disease recurrence, development of lung or bone metastases, and/or death. Patients who were alive and free of disease at the last follow-up evaluation (March 2007) were censored at that time.

Ototoxicity was evaluated using objective audiometric tests at the otorhynolaryngology consultation.

Study II: Osteosarcoma and drug transport/metabolism pathway: drug efficacy and outcome

The main clinical data for the 102 osteosarcoma patients are presented in Table 1. The median age at diagnosis was 14 years (range 3 to 34 years). At the time of diagnosis, 21% of the patients already presented metastasis, while 22% developed metastasis during follow-up. The median follow-up time was 231 months (range 3-303).

Response to treatment (necrosis) data was available for 91 patients and overall survival data were available for 101 patients. Overall survival was considered from tumour diagnosis to death. Patients who were alive at the last follow-up evaluation (January 2010) were censored at that time. EFS data were also recorded.

Table 1. Characteristics of the 102 patients with osteosarcoma from study I and II

Characteristics	*Study I* *N (%)*	Study II *N (%)*
Age at diagnosis (years)		
Mean	14.9	14.8
(minimum-maximum)	4-34	3-34
Gender		
Male	51 (56)	57 (56)
Female	40 (44)	45 (44)
Location		
Femur	46 (51)	51 (50)
Tibia	32 (35)	38 (37)
Arm	7 (7)	7 (7)
Central	6 (6)	6 (6)
Response to treatment		
Good	42 (60)	52 (57)
Poor	28 (40)	39 (43)
Metastasis		
No	57 (63)	59 (58)
At diagnosis	15 (21)	21(21)
At follow-up	19 (15)	22 (22)
Status		
Alive	69 (76)	72 (71)
Dead	22 (24)	29 (29)
Relapse		
No	76 (83)	85 (83)
Yes	15 (17)	17 (17)

1.1.2 Samples from breast cancer studies

In study III we evaluated the effect of genetic variants on treatment response in patients with locally advanced breast cancer treated with doxorubicin or docetaxel as neoadyuvant. In studies IV and V, breast cancer patients treated with capecitabine were included to identify the genetic factors related to capecitabine-induced hand-foot syndrome. Since this adverse event is common in patients treated with the drugs independently from the type of tumour, we also included a small number of capecitabine-treated colorectal cancer patients in order to increase the power of the study, considering tumour-type as a covariate when the statistical analysis was performed.

Study III: Docetaxel and doxorubicin: breast cancer treatment efficacy

A total of 186 breast cancer patients treated at the Hospital Universitario San Carlos, Madrid, Spain, were included in this study. This study was part of a clinical trial (www.clinicaltrials.gov, identifier code: NCT00123929) (Figure 5). All patients signed an informed consent form before being enrolled in the trial. The clinical characteristics of the patients are shown in Table 2.

Patients were randomly assigned to receive 4 cycles of either doxorubicin (75 mg/m^2 body-surface area as an intravenous infusion over 60 minute every 3 weeks) or docetaxel (100 mg/m^2 body-surface area as an intravenous infusion over 60 minutes every 3 weeks) as their primary therapy. After 4 cycles of chemotherapy with doxorubicin or docetaxel, patients underwent breast cancer surgery.

Clinical response was evaluated according to RECIST criteria comparing the sonographic and MRI breast assessments between pre-chemotherapy and after chemotherapy cycle four. The pathological response was evaluated in the surgery specimen. Pathological response was evaluated according to the residual cancer burden (RCB) classification of Symmans et al. (Symmans *et al*, 2007): pathological complete response (PCR) in breast and axilla, and classes I, II and III. Patients with PCR and class I were considered as having a good pathological response (good PathResp) since both have an equally good prognosis. In this study, we used the continuous variable (RCB score) which measures the amount of residual tumour burden, for the evaluation of response in the statistical analysis.

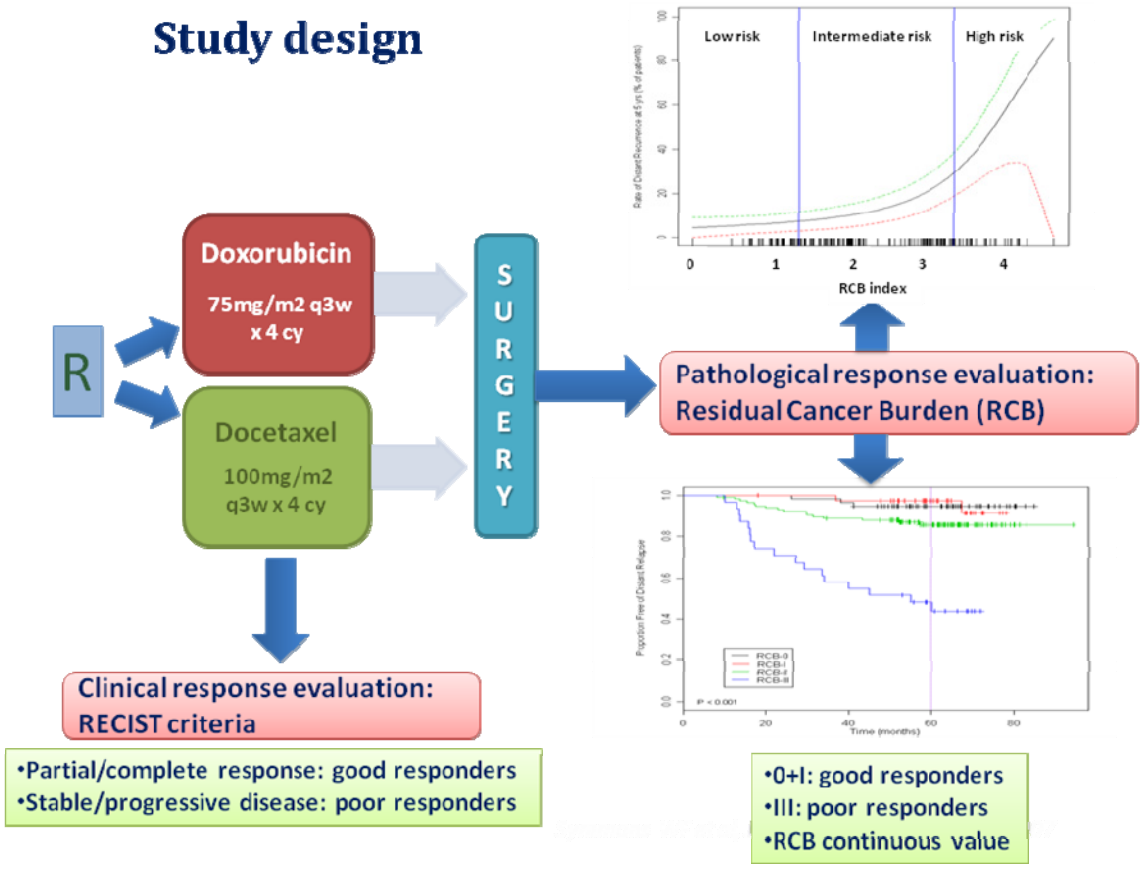


Figure 5. Study design. Breast cancer patients were randomized to receive doxorubicin or docetaxel before surgery

Table 2. Characteristics of the breast cancer patients

Characteristics	*Doxorubicin N (%)*	*Docetaxel N (%)*
Number of evaluable patients	97	85
Age		
Median	51	50
range	26-79	27-77
Tumour size; cm		
Median	6	6
range	2-15	2-15
Histology type		
Ductal	79 (81)	69 (81)
Lobular	17 (18)	10 (12)
Others	1 (1)	6 (7)
UICC stage		
II	34 (35)	31 (37)
IIIA	33 (34)	25 (29)
IIIB	30 (31)	29 (34)
Tumour grade		
3	35 (36)	36 (42)
1-2	62 (64)	49 (58)
Clinical response		
Complete response	16 (17)	14 (16)
Partial response	48 (49)	54 (64)
Stable disease	29 (30)	14 (16)
Progressive disease	4 (4)	3 (4)
Pathological response (Symmans)		
0	12 (13)	6 (7)
I	7 (7)	11 (13)
II	38 (39)	43 (51)
III	40 (41)	25 (29)
RCB		
Mean	2.63	2.40
Range	0-4.72	0-4.54

Study IV: Drug metabolism pathway and Capecitabine-induced hand-foot syndrome

We included 130 patients diagnosed with breast cancer or colorectal cancer treated at the Hospital Universitario San Carlos, Madrid, Spain, between June 2005 and March 2009 (Table 3). The study was conducted according to the declaration of Helsinki and approved by the ethics committee of the institution. All patients signed an informed consent. Most (72%) of the patients were diagnosed with breast cancer, while the remainder were patients with colorectal cancer. The median age at diagnosis was 63 years (range 28 to 88 years), and 112 patients (86%) were female.

Capecitabine was administered according to two different schedules. Colorectal cancer patients were treated with a standard regimen (1250 mg/m^2 orally every 12 hours on days 1-14 every 3 weeks), while breast cancer patients were treated either with the same standard regimen or with a continuous regimen (800 mg/m^2 orally every 12 hours daily).

Hand-foot syndrome (HFS) was graded according to the National Cancer Institute´s Common Terminology Criteria for Adverse Events (version 2). Grade 3 HFS was defined as skin changes with pain interfering with function. For study purposes, we used as study endpoint the maximum HFS grade experienced by the patients along the treatment, considering grade 0 to 2 of HFS as no or low toxicity, and grade 3 as high toxicity. Other clinical data were recorded such as age, sex, capecitabine regimen, number of reductions and hepatic metastasis (as a variable that could interfere with capecitabine metabolism).

Table 3. Characteristics of the 130 breast cancer and colorectal cancer patients treated with capecitabine included in the study IV

Characteristics	*No.*	*%*
Age at diagnosis (years)		
Mean	63	
(minimum-maximum)	28-88	
Gender		
Male	18	14
Female	112	86
Diagnostic		
Breast cancer	93	72
Colorectal Cancer	37	28
Stage		
I	3	2
II	9	7
III	33	25
IV	85	65
Treatment Setting		
Postsurgical adjuvant	39	30
First-line metastatic	26	20
Second-line metastatic	13	10
Third-line metastatic or further	52	40
Capecitabine		
Standard	104	80
Continuous	26	20
Nº Capecitabine Reductions		
0	59	45
1	54	42
≥2	17	13
Hand-foot syndrome		
Grade 0	41	31
Grade 1	23	18
Grade 2	25	19
Grade 3	41	32
Hepatic Metastasis		
No	78	60
Yes	51	40

Study V: Genome-wide association study (GWAS) and capecitabine-induced hand-foot syndrome

In this study to avoid biases due to the complexity of the classification of the phenotype (Crowley *et al*, 2009), (Turner *et al*, 2008), (Nebert, 2000a) and to increase the statistical power, only patients with extreme phenotypes (grade 0 vs grade 3 hand-foot syndrome) were enrolled.

In the discovery phase of this study, the 130 patients from Hospital San Carlos of the previous study were included, with the addition of 16 new cases collected between 2009 and 2011. An additional set of 275 capecitabine treated breast cancer and colorectal cancer patients from the Hospital Universitario Virgen de la Victoria, Malaga was collected. Only patients that didn't experience HFS, or that experienced grade 3 HFS were genotyped for the GWAS analysis, giving a final number of 163 patients (Table 4). Patients were treated as described above.

A validation set was also collected including 60 metastatic breast cancer breast cancer patients enrolled in the randomized phase II GEICAM 2009-05 clinical study of Roche comparing continuous vs intermittent capecitabine and 92 colorectal cancer patients from the Hospital General Universitario Gregorio Marañon, Madrid (Table 4). In total, 85 grade 0 or 3 HFS patients were selected for the replication study.

Table 4. Characteristics of the GWAS study populations

Characteristics	*Discovery series No.(%)*	*Validation series No.(%)*
Age at diagnosis (years)		
Mean	63	64
(minimum-maximum)	29-88	36-87
Gender		
Male	22 (13)	35 (41)
Female	141 (87)	50 (59)
Diagnostic		
Breast cancer	119 (73)	27 (32)
Colorectal Cancer	44 (27)	58 (68)
Capecitabine		
Standard	143 (88)	72 (85)
Continuous	20 (12)	13 (15)
Nº Capecitabine Reductions		
0	78 (48)	38 (45)
1	63 (39)	33 (39)
≥2	22 (13)	14 (16)
Hand-foot syndrome		
Grade 0	75 (46)	62 (73)
Grade 3	88 (54)	23 (26)

The design of the study is shown in Figure 6. All participants of the different studies gave written informed consent and the study was approved by the ethics committee of each institution.

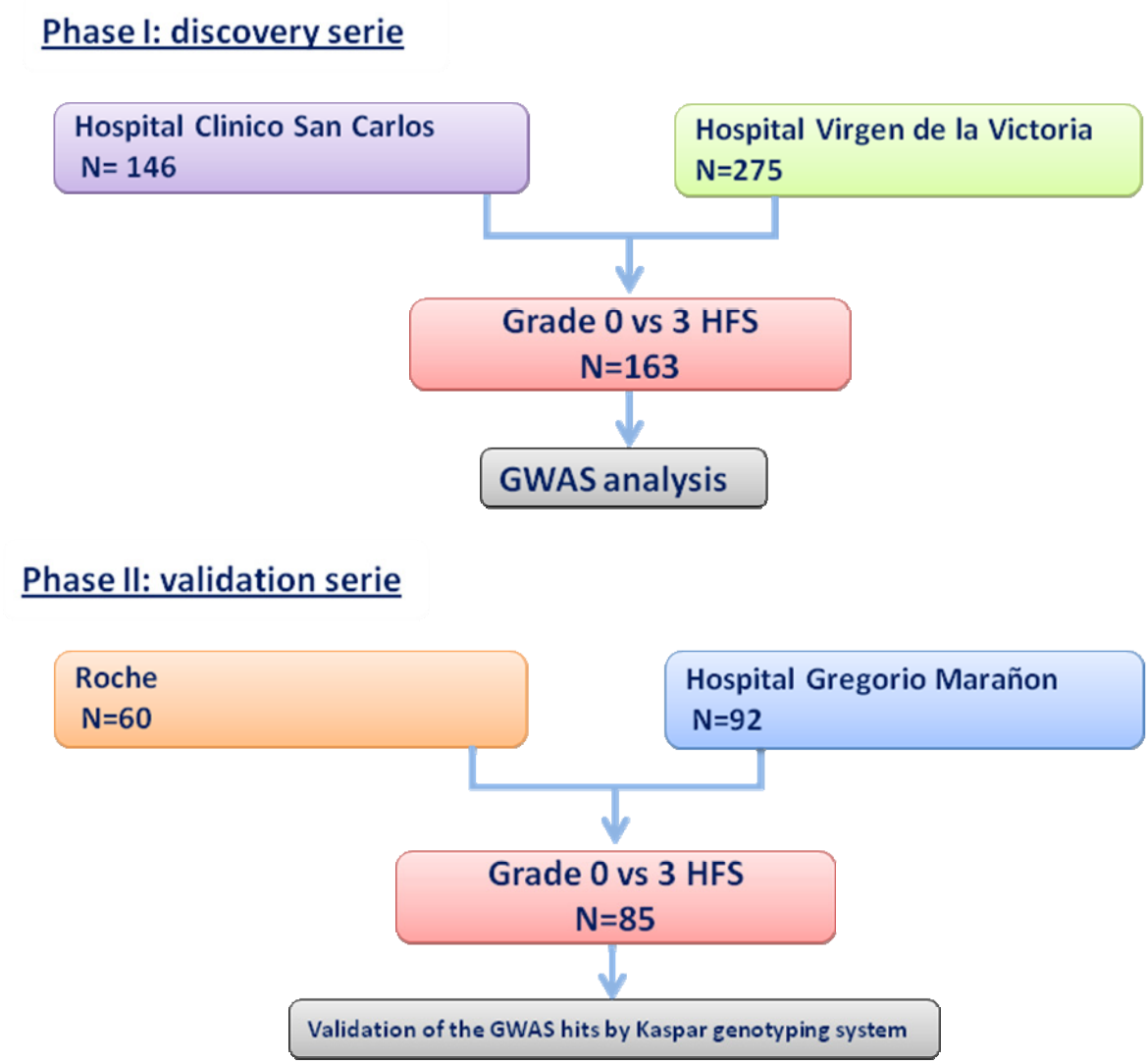


Figure 6. GWAS Patient flow. Breast cancer and colorectal cancer patients from Hospital Clinico San Carlos and Hospital Virgen de la Victoria with grade 0 or 3 HFS were included in the GWAS discovery series. Patients with the same characteristics from the Roche Clinical trial and the hospital Gregorio Marañon were included in the validation series.

1.2. Liver samples

Human liver tissue from 50 patients was obtained from the Spanish National Cancer Research Center (CNIO, Spain) Tumour Bank as non-tumourous tissue adjacent to surgically removed liver tumours or metastasis.

The study was approved by the ethics committee of the Spanish National Cancer Research Center (CNIO, Spain).
The tissues were stored at -80ºC until DNA and RNA were extracted.

1.3. Lymphoblastoid cells

Eighty-nine lymphoblastoid cell lines derived from the Caucasian Utah CEPH lines were purchased from the Coriell Institute for Medical Research (Camden, NJ). Lymphoblastoid cell lines were cultured in RPMI 1640 containing 15% fetal bovine serum (Euroclone, Siziano, Italy) and maintained in a humidified incubator at 37ºC and 5% CO2.

2. Isolation and quantification of DNA

Five to ten ml of **peripheral blood samples** were collected in tubes containing anticoagulant. Whole blood was fractionated by centrifuging during 10 minutes at 3000 rpm; the plasma upper layer was aspirated off, while the buffy coat containing the white blood cells was extracted by a Pasteur pipette. DNA was extracted from the isolated lymphocytes using standard phenol-chloroform extraction protocols for the osteosarcomas patients, while, for the other studies, DNA was extracted using automatic DNA extraction (MagNA Pure, Roche, Mannheim, Germany) according to the manufacturer's protocol.

DNA was extracted from **liver tissues** by the AllPrep DNA/RNA Mini kit (Qiagen GmbH, Hilden, Germany). Frozen tissues were disrupted and homogenized in Buffer RLT plus by passing the lysate through a 20 gauge needle fitted to a syringe. After centrifugation, the supernatant was transferred to the AllPrep DNA spin columns and centrifuged. DNA was then washed twice respectively with buffer AW1 and AW2 and eluted from the columns after the addiction of the Buffer EB.

Genomic DNA was extracted from exponentially **growing cells** using DNAZol (MBC, Molecular Research Center, Cincinnati, USA). Briefly, 1 ml of DNAzol was added to cell pellet containing to 10 million cells. After centrifugation at 10000g for 10 minutes, DNA was precipitated by the addition of 100% ethanol to the supernatant. After mixing, the DNA was removed by spooling with a pipette tip and washed twice with 75% ethanol. DNA was then dissolved in water.

In all the cases, DNA was quantified using PicoGreen (Invitrogen Corp., Carlsbad, CA). For the standard curve, a series of dilutions of genomic DNA (Clontech, Mountain View, CA 94043

USA), giving a final DNA concentration from 20 to 200 ng/µl, were prepared in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The standards and 2 µl of each sample were pipetted into a 96 well microplate (Falcon, BD Biosciences, San Jose, CA, USA). PicoGreen reagent was diluted in TE buffer according to the kit instructions and 198 µl of the mix was pipetted in the wells. The fluorescence was read at 520 nm after 480 nm excitation using DTX 800 Multimode Detector (Beckman Coulter, Fullerton, CA, USA).

3. Isolation and quantification of RNA

Total RNA was extracted from exponentially growing cells or from cryopreserved liver tissues using 1 ml TriZol (Invitrogen) and stored at -80ºC. After defreezing, the suspension was kept 10 minutes at RT and then, 200 µL of chloroform were added. Samples were mixed thoroughly 15 seconds and maintained 2-3 additional minutes at RT. After 15 minutes of centrifugation at 12000g, the aqueous phase was recovered in a fresh tube. The same volume of isopropanol was added and after vortexing, samples were kept 15 minutes at RT. Samples were centrifuged at 12,000 g, 10 minutes, at 4ºC. Pellets were washed twice with 75% cold ethanol and resuspended in RNAase free water.

One microlitre of RNA was used to measure the concentration by Nanodrop ND-1000 (Wilmington, DE, USA) and the RNA quality was tested through 1% agarose gel electrophoresis.

4. Selection of genes and polymorphisms

Genes were selected basing on the literature and on the information available at the time of each study design in the Pharmacogenomics Knowledge database PharmaGKB (www.pharmgkb.com).

The genes and polymorphisms selected for each study are illustrated below.

Study I: Osteosarcoma and DNA repair genes: treatment efficacy and ototoxicity

Eight SNPs located in six DNA repair genes involved in cisplatin-induce damage repair were selected:

- *ERCC2*: rs13181 Lys751Gln and rs1799793 Asp312Asn
- *XPC*: rs2228001 Lys939Gln
- *ERCC1*: rs3212986 Lys504Gln and rs11615 Asn118Asn

- *ERCC4*: rs744154 in intron1,
- *ERCC5*: rs1047768 His46His
- *XPA*: rs1800975 in 5'UTR.

All these SNPs have been related to platinum and radiotherapy response and/or risk of cancer in other type of tumours (Quintela-Fandino *et al*, 2006), (Park *et al*, 2001), (Gurubhagavatula *et al*, 2004), R (Ruzzo *et al*, 2007), (Vogel *et al*, 2005), (Milne *et al*, 2006), (Carles *et al*, 2006).

Study II and study III: Osteosarcoma and breast cancer drug metabolism pathways: drug efficacy and outcome

A total of 25 candidate genes reported to be involved in the metabolism or influx/efflux of the drugs used in the osteosarcoma and in the breast cancer study (cisplatin, doxorubicin, methotrexate, vincristine, cyclophosphamide and docetaxel) were selected, based on the information available in the Pharmacogenomics Knowledge database PharmaGKB (www.pharmgkb.com).

These genes encode the following proteins:

- *Transporters*: *ABCA3, ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCG2, ABCC6, SLC31A1, SLCO6A1, SLC19A1*;
- *Phase I metabolism enzymes*: *MPO, SOD1, ALDH1A1, CYP3A4, 3A5, 2A6, 2B6, 2C8, 2C19, 2C9, 1B1*;
- *Phase II metabolism enzymes: GSTM1, GSTP1* and *GSTT1*.

SNPs were selected across all these genes except for the *GSTT1* gene. CNVs were studied in the *GSTT1* and *GSTM1* genes.

SNPs were selected across all these genes in the following way:

TagSNPs for the selected genes were defined using Haploview software v.4.0 (http://www.broad.mit.edu/mpg/haploview) with an r^2= 0.8 threshold and a minor allele frequency of more than 0.05, except for the *ABCC4* gene where the number of tagSNPs was decreased to 57 due to an excessively elevated number of tagSNPs obtained according to the criteria mentioned above.

In addition, both SNPs with potentially functional effects (causing amino acid changes, potentially causing alternative splicing, in the promoter region, in putative transcription factor binding sites, or disrupting miRNAs and their targets) identified using the bioinformatics tool PupaSuite (http://bioinfo.cipf.es/pupasuite/www/index.jsp), and other functional SNPs already described in the literature were selected.

This preliminary list of candidate SNPS, was evaluated by the Assay Design Tool (ADT) which provides independent assay success prediction values, validation status and allele frequencies. The ADT generates a final_score that ranges from 0 to 1 and higher values reflect greater likelihood of success of the assay experimentally. We excluded SNPs with Final_Score lower than 0.6 (because they have lower chance of converting into functional assay and can also decrease the overall performance of all assays) and SNPs with warning codes.

A final number of 384 SNPs relevant to this study was included in an oligonucleotide pool assay for analysis using the Illumina GoldenGate Veracode technology (Illumina Inc., San Diego, CA) and two CNV using Taqman assays (Applied Biosystem)

Study IV: Metabolism pathway and capecitabine-induced hand-foot syndrome

A total of thirteen polymorphisms located in five different genes selected. Eleven polymorphisms were located in capecitabine metabolic pathway genes and the other two polymorphisms were located in the 5-FU target gene *TYMS*:

- *CES2*: rs2241409, rs11568314, rs11568311, all intronic SNPs and rs11075646 823C>G in the promoter;
- *CDD*: SNPs rs532545 -451C>T, rs602950 -92A>G, both located in the promoter, and the coding SNP rs2072671, Lys27Gln (Ribelles *et al*, 2008), (Fitzgerald *et al*, 2006), (Sugiyama *et al*, 2007), (Fukunaga *et al*, 2004), (Gilbert *et al*, 2006);
- *DPD*: the intronic SNP rs3918290, IVS14+1G>A, in the splice donor site flanking exon 14 that causes exon skipping and inactivation of DPD allele (van Kuilenburg, 2004), (van Kuilenburg *et al*, 2001), (Salgado *et al*, 2007), (Wei *et al*, 1996);
- *TP*: the intronic SNP rs470119 and the coding SNPs rs11479 Ser471Leu and rs131804 Ala324Ala.;

- *TYMS*: a 28bp double or triple tandem repeat, including a G>C mutation in the 5' region and a 6bp deletion in the 3' region (Ulrich *et al*, 2002), (Salgado *et al*, 2007), (Mandola *et al*, 2003) .

All polymorphisms have been described in the literature, as possible functional variants except in the TP and CES2 genes, for which, due to the lack of candidate functional SNPs, tagSNPs were selected as described above.

5. Genotyping

5.1. TaqMan SNP Genotyping System

SNPs that were genotyped using TaqMan SNP Genotyping System (Applied Biosystems, Foster City, CA, USA) are shown in Table 5.

The TaqMan Genotyping assays contain:

- Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest.
- Two TaqMan probes, one labelled with VIC dye to detect the Allele 1 and one probe labelled with FAM dye to detect the Allele 2 sequence. All probes include a quencher so that there is no fluorescence emission.

During the first step of a TaqMan SNP Genotyping Assay experiment, AmpliTaq Gold® DNA polymerase from the TaqMan Genotyping Master Mix, amplifies target DNA using sequence-specific primers. Detection is achieved with proven 5' nuclease chemistry by means of exonuclease cleavage of a 5' allele-specific dye label, which generates the permanent assay signal (Figure 7).

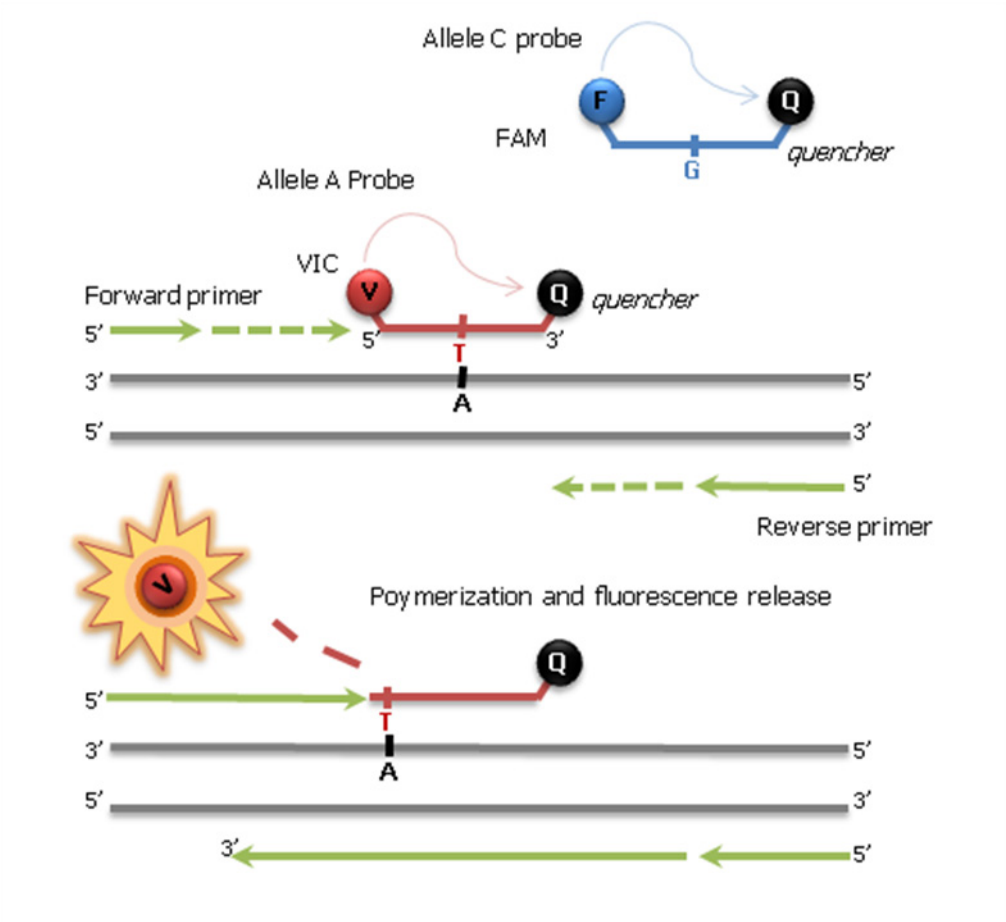


Figure 7. TaqMan Genotyping System

Ten ng of DNA were used for each reaction, and combined with 2.5 ul of 2X TaqMan Genotyping Master Mix, 0.25 ul of 20X Taqman SNP genotyping assay, and water to a final reaction volume of 5 ul. The amplification conditions consisted of an initial step at 95 ºC for 10 min, followed by 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC.

The sequence Detection System 7900HT (Applied Biosystems) was used for fluorescence detection and allele assignment. The Sequence Detection System (SDS) Software (version 2.2.2) uses the fluorescence measurements made during the plate read to plot fluorescence (Rn) values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample (figure 8). Coriell sample/s in duplicate (Coriell Cell Repository, Camden, NJ) as positive controls (i.e. DNAs with known genotypes) and NTC (no template control) were included in all assays.

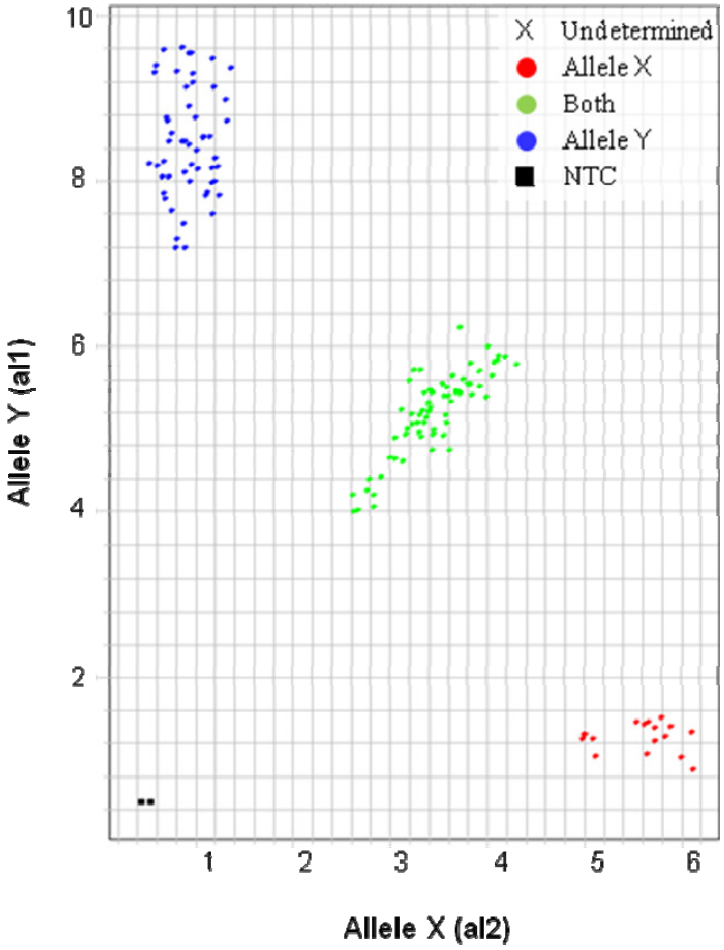


Figure 8. Taqman and KASPar SNP allelic discrimination. Genotypes were assigned by allelic discrimination with the Sequence Detection System 7900HT.

5.2. KASPar SNP Genotyping System

SNPs that were genotyped using KASPar SNP Genotyping System (Kbiosciences, Herts, UK) are shown in Table 5.

The Kaspar genotyping system is based on:

- two allele-specific primers (one for each SNP allele). Each primer has an unlabelled tail sequence at the 5' end and the two primers have different tail sequences
- a common reverse primer
- two fluor-labelled oligos complementary to the sequences of the tails of the allele-specific primers. This oligos are labelled one with a fluorochrome (FAM or VIC) and with a quenchers so that there is no fluorescence emission.

In the beginning of the PCR, the appropriate allele-specific primer and the common primer binds to the DNA at the SNP locus and PCR occurs. As PCR proceeds further, the fluor-labelled oligo becomes incorporated into the template as well, and is hence no longer bound to its

quencher-labelled complementary oligo. As the fluorescence is no longer quenched, the appropriate fluorescent signal is generated and detected by the usual means (Figure 9).

If the genotype of a diploid individual for a particular SNP is homozygous, only a FAM OR VIC signal will be generated. If the individual is heterozygous, a FAM AND VIC signal will be generated.

Ten ng of DNA were used for each reaction, and combined with 2 ul of 2X KASPar Reaction Mix, 0.055 ul of KASPar genotyping assay, MgCl2 to a final concentration of 2.2 mM and water to a final reaction volume of 4 ul. The amplification conditions consisted of an initial step at 94 ºC for 15 min, followed by 20 cycles of 10 s at 94 ºC, 5 s at 57ºC and 10s at 72 ºC, and then by 18 cycles of 10 s at 94 ºC, 20 s at 57ºC and 40s at 72 ºC. The same internal controls and the same detection system used for Taqman assays were used as described above.

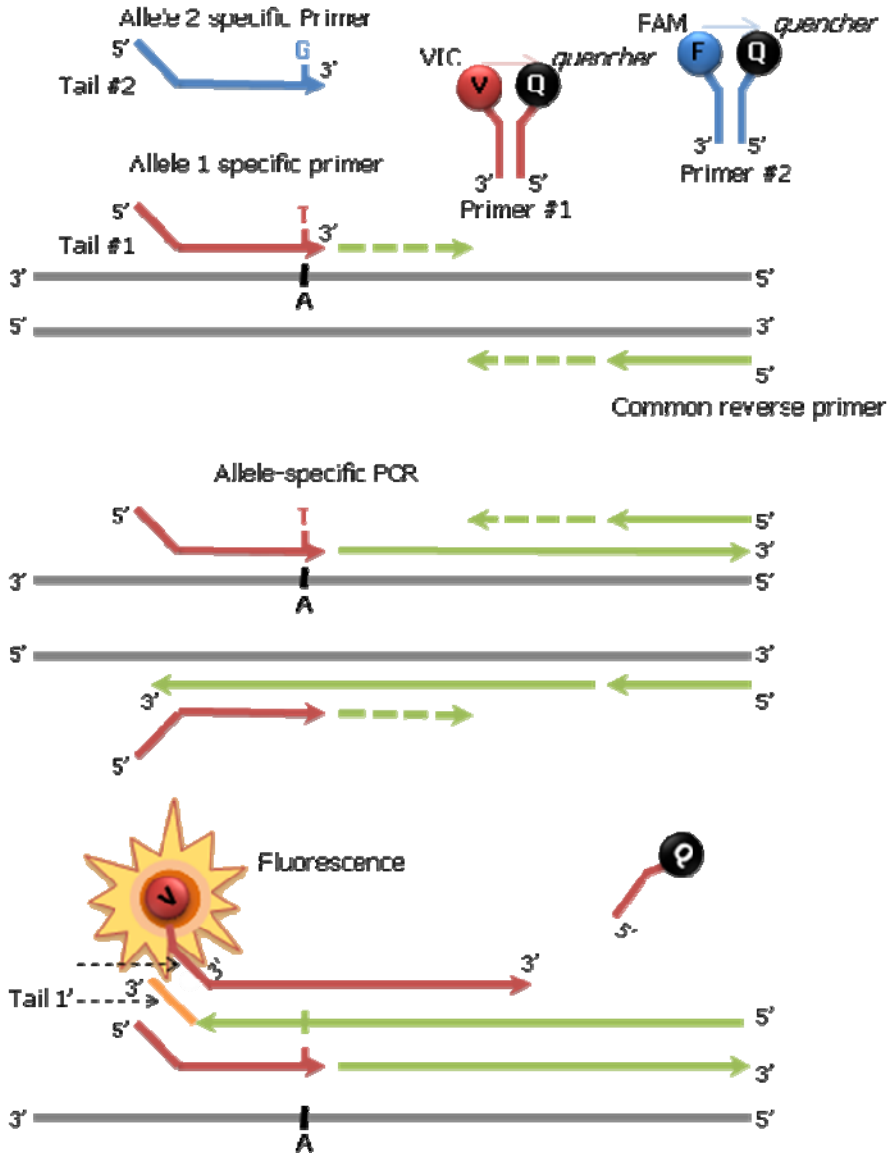


Figure 9. KASPar SNP Genotyping System

Table 5. Primers for genotyping by Taqman and Kaspar techniques

SNP	*Change*	*Genotyping method*
rs3212986	G/T	TaqMan
rs11615	T/C	TaqMan
rs13181	T/G	TaqMan
rs1799793	G/A	TaqMan
rs2228001	A/C	TaqMan
rs744154	C/G	TaqMan
rs1047768	C/T	TaqMan
rs1800975	G/A	KASPar
rs532545	C/T	KASPar
rs602950	A/G	KASPar
rs2072671	A/C	KASPar
rs3215400	-/C	KASPar
rs603412	C/G	KASPar
rs2241409	C/T	KASPar
rs11568314	A/T	KASPar
rs11568311	A/G	KASPar
rs3918290	A/G	KASPar
rs470119	A/G	KASPar
rs11479	C/T	KASPar
rs131804	A/G	KASPar
rs2231137	A/G	KASPar
rs2619170	A/G	KASPar
rs1121941	G/T	KASPar
rs2755087	C/T	KASPar
rs2237827	C/T	KASPar
rs2237826	C/T	KASPar
rs6093063	G/T	KASPar
rs12221182	C/T	KASPar
rs9573665	C/T	KASPar
rs531790	A/G	KASPar
rs7279195	A/G	KASPar
rs7282914	G/T	KASPar
rs730354	A/G	KASPar
rs17337019	A/C	KASPar
rs9593132	C/T	KASPar

5.3. Restriction Fragment Length Polymorphism (RFLP)

For the polymorphism in the 3'UTR of TYMS gene, restriction fragment length polymorphism (RFLP) was used. Primers were designed with web programme Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). A fragment containing the 6bp deletion was amplified starting from 100 ng genomic DNA using the following primers: 5'-CAAATCTGAGGGAGCTGAGT -3' and 5'- CAGATAAGTGGCAGTACAGA -3'. PCR products were then digested with 15 ul of PCR product 2 U of the DraI restriction enzyme (Fermentas Inc.,

Hanover, MD, USA), the supplied buffer to 1X in a total volume of 20 ul and incubated for 2 hours at 37ªC. Expected fragments sizes were separated (a fragment of 142 bp for the deleted allele and two fragments of 88 and 60 bp for the inserted allele) on a 3% agarose gel.

5.4. Sequencing

For the polymorphism in the 5'UTR of TS gene direct sequencing was used. Primers were designed as described above. A fragment containing the 28bp repeat was amplified starting from 100 ng genomic DNA using the following primers: 5'-TTCCCGGGTTTCCTAAGACT-3' and 5'-TGGATCTGCCCCAGGTACT-3'.

For the fine mapping at the *CDD* promoter, A 959 bp fragment of the promoter was amplified from 30 healthy controls using the following primers: 5'-ATGCAGTGGTGCAATCTGAG-3' and 5'-GTGCCCACCTTTACCTTTGA-3'.

For the genotyping of rs717620 in *ABCC2* gene, a 516 bp fragment was amplified from 30 frozen liver tissues using the following primers: 5'-CATGTCCATCCACTGTTTCAATGTA-3' and 5'-TGGAAGGTTTTTACCTGTTTCTCTTTAG-3'.

PCR products were purified by ExoSap-IT (USB Corporation, Ohio, USA) and directly by Sanger method with sequencer 3730 from Applied Biosystems.

5.5. GoldenGate Veracode technology

Illumina GoldenGate technology is based on allele-specific primer extension (ASPE) assays (Figure 10). Briefly, in the first step DNA is activated to enable binding to paramagnetic particles and then hybridized with the assay oligonucleotides, hybridization buffer, and paramagnetic particles. Three oligonucleotides are designed for each SNP locus. Two oligos are specific to each allele of the SNP site, called the Allele Specific Oligos(ASOs). A third oligo, the Locus Specific Oligo (LSO), hybridizes several bases downstream from the SNP site. All three oligonucleotide sequences contain universal PCR primer sites; the LSO also contains a unique address sequence that targets a particular VeraCode Bead type. After hybridization, extension of the appropriate ASO and ligation of the extended product to the LSO are performed. The resultant ligation products serve as the PCR templates with universal PCR primers P1, P2, and P3. Universal primers P1 and P2 are Cy3 and Cy5 labelled to allow discrimination of the two alleles. PCR products are hybridized to their complement bead type through their unique address sequences. The BeadXpressTM Reader is used for code identification and fluorescent signal detection. During scanning, a laser excites the cylindrical glass microbeads that carry

high-density codes that. Data generated from theBeadXpress Reader are analyzed using the Illumina GenomeStudio software for automated genotype clustering and calling.

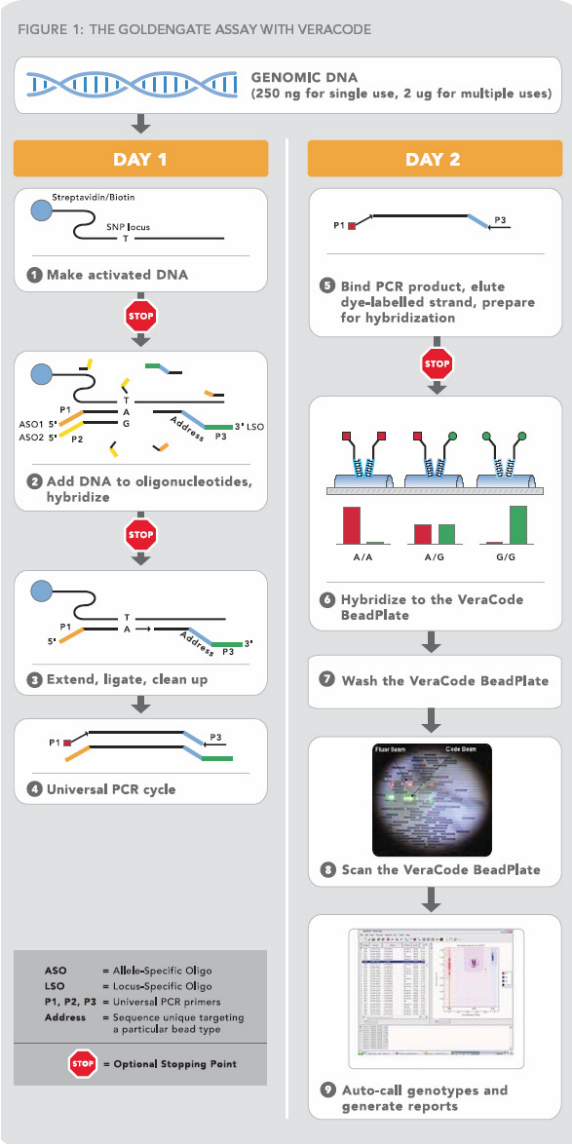


Figure 10. Illumina Veracode Goldengate protocol (from Illumina website, www.illumina.com)

250-300 ng of DNA for each sample were genotyped using the GoldenGate Genotyping Assay with Veracode technology according to the published Illumina protocol. Coriell samples in duplicate and trios (Coriell Cell Repository, Camden, NJ) were included across the plates. One NTC per plate was also genotyped.

5.6. GSTM1 and GSTT1 copy number assays

GSTT1 and *GSTM1* copy number was calculated using Taqman Copy Number Assays (Hs00010004_cn probe for *GSTT1* and Hs02575461_cn for *GSTM1*, Applied Biosystems, Foster City, CA) following the manufacturer's protocol on an ABI PRISM 7900 Sequence Detection

System (Applied Biosystems). The *RNAse P*, a sequence known to exist in two copies in a diploid genome, was used as reference for copy number quantification.

Ten ng of DNA were used for each reaction, and combined with 5 ul of 2X TaqMan Universal PCR Master Mix (No AmpErase UNG), 0.5 ul of 20X Taqman Copy Number assay, containing two primers and a FAM dye-labeled MGB probe, 0.5 ul of TaqMan Copy Number Reference Assay, containing two primers and a VIC dye-labeled TAMRA probe and water to a final reaction volume of 10 ul.

The amplification conditions consisted of an initial step at 95 ºC for 10 min, followed by 40 cycles of 15 s at 95 ºC and 1 min at 60 ºC. Positive controls (i.e. DNAs with known number of copies) were included in all assays.

Data were analyzed using absolute quantification of resulting Ct values generated on the sequence detection system. Copy number was estimated using the CopyCaller 1.0 software (Applied Biosystems). Each sample was evaluated in triplicate.

5.7. Whole Genome genotyping by Infinium assay

Genome-wide genotyping was performed using the Infinium assays (Illumina, San Diego, CA). In this system a whole-genome amplification step is used to increase the amount of DNA up to 1000-fold. The DNA is fragmented and captured on BeadChips by hybridisation to immobilised SNP-specific primers, followed by single-base primer extension (Figure 11). Single-base extension of the oligos on the BeadChip, using the captured DNA as a template, incorporates detectable labels on the BeadChip and determines the genotype call for the sample. If there is a perfect match, extension occurs and signal is generated. If there is a mismatch, extension does not occur and no signal is generated.

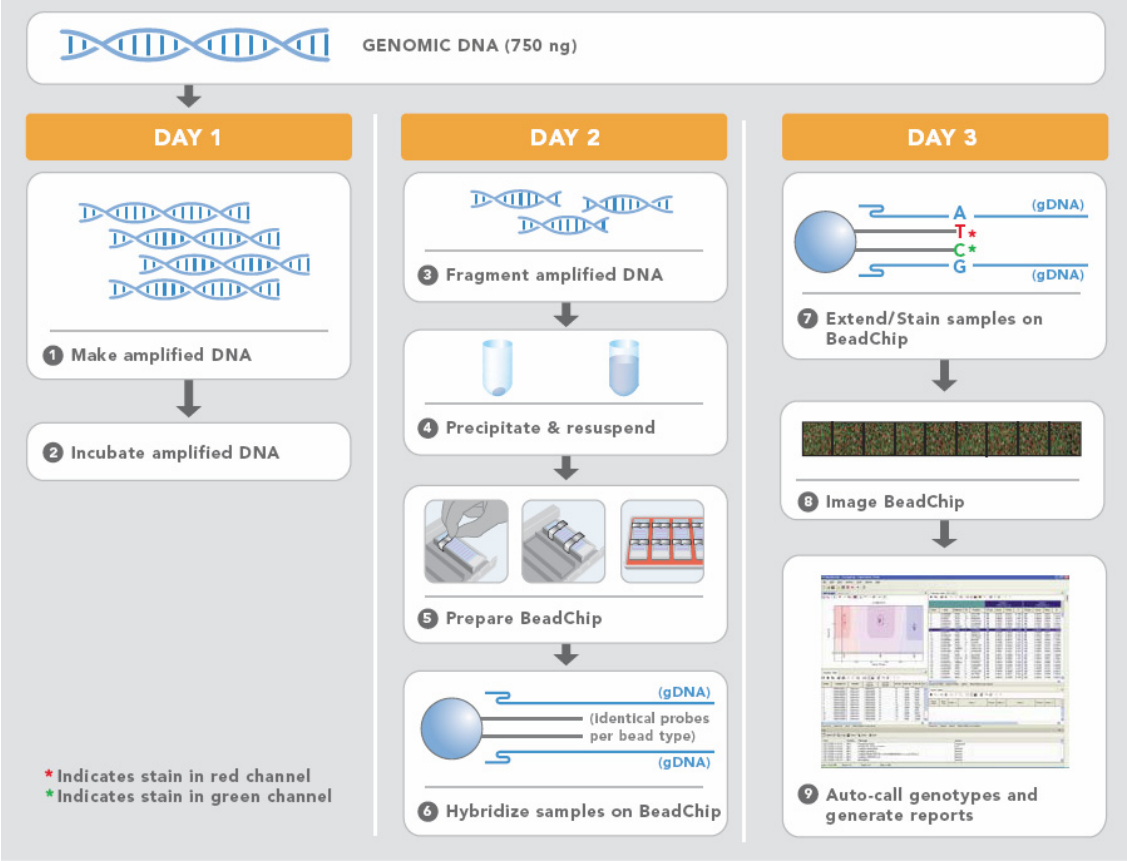


Figure 11. Infinium assay protocol (from Illumina website, www.illumina.com)

BeadChips are imaged using the Illumina iScan System, a two-channel high-resolution laser imagers that scan BeadChips at two wavelengths simultaneously and create an image file for each channel (i.e., two per array). The GenomeScan software determines intensity values for each bead type and creates data files for each channel. The GenomeStudio software package extracts whole-genome DNA analysis data from image data files created by the Illumina BeadArray Reader.

The analysis focused on 616795 markers. 163 samples, of which 4 were genotyped in duplicate, were genotyped applying 250 ng of DNA to the Human 610-Quad chip (Illumina, San Diego, CA) following the manufacturer's instructions. After filtering (see genotyping quality control section next), 520871 SNPs were successfully analyzed.

6. Genotyping quality control (QC)

Coriell samples in duplicate and trios from Coriell (Coriell Cell Repository, Camden, NJ) were genotyped across the plates in all the genotyping methods used in this thesis.

The following criteria were followed for SNPs exclusion:

- SNPs showing Mendelian allele-transmission errors or showing discordant genotypes were excluded from the analysis.
- SNPs with call rates <0.95 were excluded.
- SNPs that deviated from Hardy-Weinberg equilibrium (HWE) or with MAF<5% were removed from the analysis.

After SNP exclusion, sample call rate was calculated and samples with call rates <0.95 were excluded from further analysis.

In addition, internal controls provided by the GoldenGate and Infinium assays that can be visualized in GenomeStudio Software are checked. These include sample-dependent, sample-independent and contamination controls that provide relevant information about the overall performance of the reagents, samples and equipment used in the experiment.

7. Gene expression analysis

7.1. Real time quantitative PCR (qRT-PCR)

1 ug of RNA was reverse transcribed using an oligo random hexamers and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations .
mRNAs were quantified by real-time PCR with the Sequence Detection System 7900HT (Applied Biosystems), using the TaqMan Gene Expression Assays (Applied Biosystems), (Hs00156401_m1 probe for CDD , Hs00184491 for ABCB1, Hs00166123_m1 for ABCC2 (si ponemos esta parte) and Hs9999905_m1 for the GAPDH gene, used as reference).
2.4 ul of a 1/10 cDNA dilution were used for each reaction, and combined with 6 ul of 2X TaqMan Universal PCR Master Mix (No AmpErase UNG), 0.6 ul of 20X Taqman Gene Expression assay, and water to a final reaction volume of 12 ul.
The amplification conditions consisted of an initial step at 95 ºC for 10 min, followed by 55 cycles of 15 s at 95 ºC and 1 min at 60 ºC. The GAPDH transcript level was used as a reference.

Negative controls were present in all series of PCRs and all assays were carried out in triplicates.

Data were analyzed using absolute quantification on resulting Ct values generated on the sequence detection system. Serial 1/2 dilutions of cDNA of a control sample were used to generate standard curves, and quantity mean (Qty mean) for each sample was calculated.

7.2. Allele-specific expression assay

Allele-specific mRNA expression was measured amplifying a 244 bp region around SNP rs3215400 in the 43 Caucasian lymphoblastoid cell lines heterozygous for this SNP. The following primers were used: 5'-AAAGCTGCGTACCTGAGAGC-3' and 5'-TGACTGTAGGGGCAGTAGGC-3'. cDNA was prepared from RNA treated with DNAase I (Ambion, Austin, TX) using Superscript II Reverse Transcriptase (Invitrogen) with random hexamers according to the manufacturer's recommendations. Primer extension with fluorescent dideoxynucleotides was performed with the following primer: 5'-TTTTTTTTGCCGGAGCTCCTGTTTCC-3' using the SNaPshot system (Applied Biosystems), and followed by capillary electrophoresis on an ABI3730 DNA analyzer (Applied Biosystems). Data were analyzed by Peak Scanner 1.0 software (Applied Biosystems). Allele-specific expression (ASE) ratios were calculated as follows: the ratio between the two alleles peak area of cDNA was divided by the same ratio for genomic DNA. Each sample was assayed using two independent cDNA preparations; two independent single base extensions were run for each cDNA preparation, giving a total of four replicates. The SNaPshot ASE variation value for each individual is given as the average of the four analyses.

8. Statistical analysis

The PLINK and R (version 2.6.0.2) software were used for all analyses, while the SPSS software (version 15.0, SPSS Inc., Chicago, IL, USA) was used for generating Kaplan-Meier curves and the Mann-Whitney t-test. Haplotypes were inferred by PHASE software, version 2.0 .

Associations between genotypes or haplotypes and discrete variables were assessed using logistic regression analysis (Hosmer DW, 2000), comparing genotype frequencies in each category and estimating odds ratios (OR). Homozygotes for the most frequent allele were used as the reference group. In addition to the model comparing the genotypes separately (co-dominant model), we considered log-additive, dominant, and recessive models.

Association between genotypes and continuous variable RBC index scores was assessed by U Mann-Whitney/t-student test.

SNPs were assessed in relation to overall survival and event-free survival using Cox regression analysis (Hosmer DW, 1999).

In particular, for the GWAS data, SNPs were assessed in relation to the cumulative doses of drug using Cox regression analysis. The PLINK software was used modeling the cumulative doses of capecitabine up to the event being in this case considered the development of grade 3 hand-foot syndrome. Patients with no HFS were censored at total cumulative doses.

Associations between genotypes and gene expression data were assessed by ANOVA when considering the three genotypes separately, and Student's t-test assuming equal variances considering the wild type homozygous versus the polymorphic heterozygous and homozygous genotypes. All expression data (Qty mean) were log2 transformed to obtain normally distributed data.

A permutation test was used to estimate *p-values* corrected for multiple testing. Each replicate consisted of randomly assigning the set of three variables, response to treatment, survival status and analysis time, across subjects and then carrying out the association analyses for each of the successfully genotyped variants and each of the two outcomes (response to treatment and survival). The minimum *p-value* of these tests was then recorded. Ten thousand such replicates were carried out and the corrected *p-values* were estimated as the proportion of replicate *p-values* less than the corresponding unadjusted *p-value*. Reported *p-values* are uncorrected for multiple testing, unless otherwise stated. Only SNP associations with corrected *p-values* <0.05 were considered statistically significant.

Excepcionally GWAS *p-values* were not corrected for multiple testing and uncorrected *p-values* smaller than $1x10^{-5}$ were considered to be analysed in an independent series of patients for replication analysis.

9. *In silico* prediction

We performed in silico prediction of putative transcription factor binding sites using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html, v1.3) and Jaspar (http://jaspar.cgb.ki.se/).

RESULTS

Results, part I

Pharmacogenetics of Osteosarcoma treatment

1.1 Study I: Osteosarcoma and DNA repair genes: treatment efficacy and ototoxicity

Cisplatin is one of the most effective chemotherapeutic agents used for osteosarcoma treatment. Since the Nucleotide Excision Repair (NER) pathway is responsible for the removal of DNA adducts induced by platinum compounds, we analyzed the association between treatment response and polymorphisms in the NER genes *ERCC2, XPC, ERCC1, ERCC4, ERCC5*, and *XPA* in osteosarcoma patients treated with cisplatin.

A total of eight SNPs were finally genotyped in 70 osteosarcoma patients treated with cisplatin and analyzed in association with tumour response and event-free survival.

None of the clinical variable associated with tumour response or with EFS.

1.1.1 ERCC2 rs13181 and XPC rs2228001 polymorphisms are associated with tumour response

The SNPs analyzed and the results of association with tumour response to treatment are shown in Table 6.

A significant association was detected for the presence of at least one polymorphic allele of each of rs13181 (Lys751Gln) in *ERCC2* and rs2228001 (Lys939Gln) in *XPC*. In particular, the polymorphic G allele of Lys751Gln was associated with a poor response: the estimated odd ratio (OR) under a dominant model was 4.89 (95%CI=1.64-14.54, *p-value*= 0.004). We found that only 45% (18 of 40) of patients with at least one G allele were good responders compared to 80% (24 of 30) of patients homozygous for the T allele. On the contrary, the polymorphic C allele of *XPC* rs2228001 was significantly associated with good response (OR=0.34, 95%CI=0.12-0.91, *p-value*=0.032). For this SNP, 71% (29 of 41) of carriers of at least C allele responded to therapy compared to 45% (13 of 29) of patients homozygous for the A allele.

Only the association with *ERCC2* rs13181 polymorphism was maintained after correction for multiple testing (adjusted *p-value*=0.047). No evidence of association was found for the other polymorphisms in the NER genes considered.

Table 6. Logistic regression analysis assessing associations of polymorphisms with tumour response

Genotypes	No (%)	Odd ratio	95% CIs	*P-value*
ERCC2 rs13181				
TT	39 (42.9)	Referent		
TG	40 (44.0)	5.54	1.76-17.39	.003
GG	12 (13.2)	3.20	0.65-15.70	.152
TG/GG		**4.89**	**1.64-14.54**	**.004**
ERCC2 rs1799793				
GG	39 (42.9)	Referent		
AG	42 (46.2)	1.52	0.54-4.28	.431
AA	10 (11.0)	3.50	0.69-17.64	.129
AG/AA		1.80	0.67-4.80	.241
XPC rs2228001				
AA	36 (39.6)	Referent		
AC	43 (47.3)	0.28	0.10-0.84	.023
CC	12 (13.2)	0.54	0.13-2.34	.411
AC/CC		**0.34**	**0.12-0.91**	**.032**
ERCC1 rs3212986				
GG	50 (54.9)	Referent		
GT	30 (33.0)	1.64	0.56-4.78	.362
TT	11 (12.1)	2.51	0.63-10.05	.194
GT/TT		1.87	0.71-4.94	.206
ERCC1 rs11615				
TT	29 (31.9)	Referent		
CT	42 (46.2)	1.37	0.43-4.32	.593
CC	20 (22.0)	1.78	0.48-6.62	.391
CT/CC		1.50	0.51-4.37	.457
ERCC4 rs744154				
CC	41 (45.1)	Referent		
CG	44 (48.4)	1.23	0.45-3.38	.692
GG	6 (6.6)	2.70	0.38-18.96	.318
GG		2.40	0.37-15.38	.356
ERCC5 rs1047768				
CC	23 (25.3)	Referent		
TC	53 (58.2)	0.44	0.14-1.37	.157
TT	15 (16.5)	0.34	0.07-1.77	.201
TC/TT		0.42	0.14-1.26	.123
XPA rs1800975				
GG	45 (49.5)	Referent		
AG	38 (41.8)	0.75	0.27-2.09	.577
AA	8 (8.8)	0.76	0.16-3.70	.734
AG/AA		0.75	0.29-1.96	.556

1.1.2 ERCC2 rs13181 polymorphism is associated with event-free survival

The polymorphic G allele of rs13181 was significantly associated with shorter EFS (Hazard Ratio (HR) under a dominant model =5.76, 95%CI=1.30-25.55, *p-value* =0.021) (Table 7 and Figure 12). The median EFS of patients that carried the GG genotype was 141 months compared to 240 months for TT homozygotes.

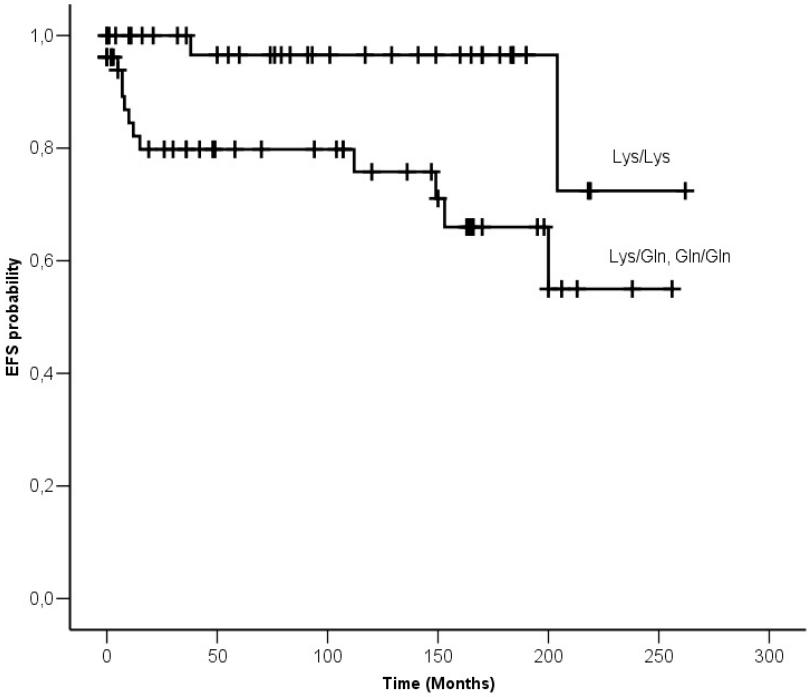


Figure 12. ERCC2 rs13181 effect on event-free survival. Kaplan-Meier curves for event-free survival of osteosarcoma patients treated with platinum-based therapy. Analysis for *ERCC2* rs13181 (X^2 =6.82, *p-value*=0.009)

Table 7. Cox regression analysis assessing associations of polymorphisms with event-free survival

Genotypes	No (%)	Hazard ratio	95% CIs	*P-value*
ERCC2 rs13181				
TT	39 (42.9)	Referent		
TG	40 (44.0)	5.06	1.09-23.46	.038
GG	12 (13.2)	8.33	1.52-45.56	.014
TG/GG		**5.76**	**1.30-25.55**	**.021**
ERCC2 rs1799793				
GG	39 (42.9)	Referent		
AG	42 (46.2)	1.71	0.50-5.87	.390
AA	10 (11.0)	3.83	0.95-15.47	.059
AG/AA		2.14	0.68-6.74	.194
XPC rs2228001				
AA	36 (39.6)	Referent		
AC	43 (47.3)	1.05	0.33-3.33	.931
CC	12 (13.2)	1.12	0.26-4.80	.875
AC/CC		1.07	0.36-3.16	.900
ERCC1 rs3212986				
GG	50 (54.9)	Referent		
GT	30 (33.0)	2.43	0.76-7.77	.134
TT	11 (12.1)	2.05	0.49-8.65	.328
GT/TT		2.30	0.78-6.75	.129
ERCC1 rs11615				
TT	29 (31.9)	Referent		
CT	42 (46.2)	2.80	0.59-13.20	.193
CC	20 (22.0)	3.35	0.64-17.48	.151
CT/CC		2.98	0.67-13.27	.151
ERCC4 rs744154				
CC	41 (45.1)	Referent		
CG	44 (48.4)	0.51	0.18-1.45	.886
GG	6 (6.6)	.00	.00	.977
GG		0.04	.00-297.11	.978
ERCC5 rs1047768				
CC	23 (25.3)	Referent		
TC	53 (58.2)	0.57	0.18-1.76	.327
TT	15 (16.5)	0.55	0.11-2.84	.474
TC/TT		0.57	0.19-1.67	.301
XPA rs1800975				
GG	45 (49.5)	Referent		
AG	38 (41.8)	1.90	0.62-5.83	.261
AA	8 (8.8)	2.27	0.43-12.00	.333
AG/AA		1.96	0.67-5.78	.220

1.1.3. XPC rs2228001 polymorphism is associated with ototoxicity

Data on ototoxicity were available for 32 patients. We detected a marginally significant association between this specific type of toxicity and rs2228001 (*XPC*). Ototoxicity was observed in 27% in patients with the AA genotype compared to 80% in patients with the CC genotype (OR = 17.16, 95% CI=1.10-266.8 *p-value*=0.042, Table 8). The frequencies of the AA, AC and CC genotypes in this SNP were 20%, 53% and 27% respectively in 15 patients with ototoxicity and 47%, 47% and 6% respectively in the 17 patients without hearing impairment. Sixty-seven percent of the patients with ototoxicity were good responders.

Table 8. Logistic regression analysis for association of XPC Lys939Gln polymorphism with ototoxicity

% of Patients with ototoxicity		Logistic regression		
		Odd Ratio	95% CIs	*P-value*
XPC rs2228001				
AA	27% (3 of 11)	Referent		
AC	50% (8 of 16)	3.62	0.56-23.60	.179
CC	80% (4 of 5)	17.16	1.10-266.78	.042

1.2. Study II: Osteosarcoma and drug transport/metabolism pathway: drug efficacy and outcome

In this study, we studied a comprehensive set of SNPs and CNVs that characterize the genetic variation of the multiple metabolic and transport pathways of drugs used in osteosarcoma treatment and their association with drug response and survival. We screened 102 osteosarcoma patients for 366 Single Nucleotide Polymorphisms (SNPs) and 2 copy number variants (CNVs) in 24 genes involved in the metabolism or transport of cisplatin, doxorubicin, methotrexate, vincristine, and cyclophosphamide. We studied the association of the genotypes with tumour response and survival.

After QC, a total of 346 SNPs out of 366 and two CNVs were successfully analyzed. Eleven patients were removed for low genotyping call rate (< 95%), so finally 91 patients were successfully analyzed.

Of the clinical variables analyzed, metastasis at diagnosis was found to be associated with increased risk of death (HR=2.92, 95%CI=1.35-6.28, *p-value*=0.006).

1.2.1. An ABCC3 polymorphism associates with overall survival and event-free survival

The T allele of the synonymous SNP rs4148416 (G1013G) in the *ABCC3* gene was associated with higher risk of death (per-allele HR=8.14, 95%CI=2.73-20.2, *p-value*=5.1x10^{-5}), (Table 9 and Figure 13A). In particular, the five-years relative survival rate for patients carrying the CC genotype of rs4148416 was 78% compared to 20% for heterozygous patients. The same result was obtained when considering EFS.

Table 9. *ABCC3* polymorphism associated with overall survival (OS) and event-free survival (EFS)

SNP	Genotype	N	5-year survival rate	OS HR* (95%CI) *P-value*	OS Adjusted**HR (95%CI) *P-value*	EFS HR (95%CI) *P-value*
rs4148416	CC	85	78%			
	CT	5	20%			
	per allele T			8.14	7.25	6.33
				(2.73-20.2)	(2.62-20.1)	(1.79-12.7)
				0.000051	0.00014	0.00028

1.2.2. ABCB1 polymorphisms associate with overall survival and event-free survival

Rs4148737, an intronic SNP located in the *ABCB1* gene, was associated with poorer overall survival (per-allele HR=3.66, 95%CI=1.85-6.11, *p-value*=6.9x10^{-5}) (Table 10 and Figure 13B). The 5-years survival rate for patients carrying the common AA genotype was 93% compared to 38% for patients homozygous for the G allele.

The minor alleles of two other SNPs (rs1128503 and rs10276036) in this gene, in complete linkage disequilibrium (LD, r^2=1.0), and in partial LD with rs4148737 (r^2=0.55) (Figure 14), were also associated with better overall survival (per-allele HR=0.24, 95%CI=0.11-0.47, *p-value*=7.9x10^{-5}). (Table 10 and Figure 13C). For these two SNPs, the estimated five-year survival rate for common homozygotes was 49% compared to 100% for patients homozygous for the rare allele). These results did not change substantially after adjusting for metastasis at diagnosis.

Table 10. Five-years survival rate for genotypes of ABCC3 and ABCB1

SNP	Genotype	N	5-year **survival rate**	OS HR* **(95%CI)** ***P-value***	OS Adjusted**HR **(95%CI)** ***P-value***	EFS HR **(95%CI)** ***P-value***
rs4148737	AA	28	93%			
	AG	49	75%			
	GG	13	38%			
	per allele G			3.66	2.83	2.60
				(1.85-6.11)	(1.56-5.12)	(1.24-3.22)
				0.000069	0.00061	0.00051
rs1128503	CC	26	49%			
	CT	50	82%			
	TT	14	100%			
	per allele T			0.24	0.27	0.42
				(0.11-0.47)	(0.13-0.54)	(0.29-0.81)
				0.000079	0.00023	0.0021
rs10276036	TT	26	49%			
	TC	50	82%			
	CC	14	100%			
	per allele C			0.24	0.27	0.42
				(0.11-0.47)	(0.13-0.54)	(0.29-0.81)
				0.000079	0.00023	0.0021

We also studied these SNPs in relation to event-free survival (EFS) and highly consistent results were observed (Table 10).

None of the SNPs analyzed were significantly associated with tumour response after correction for multiple testing.

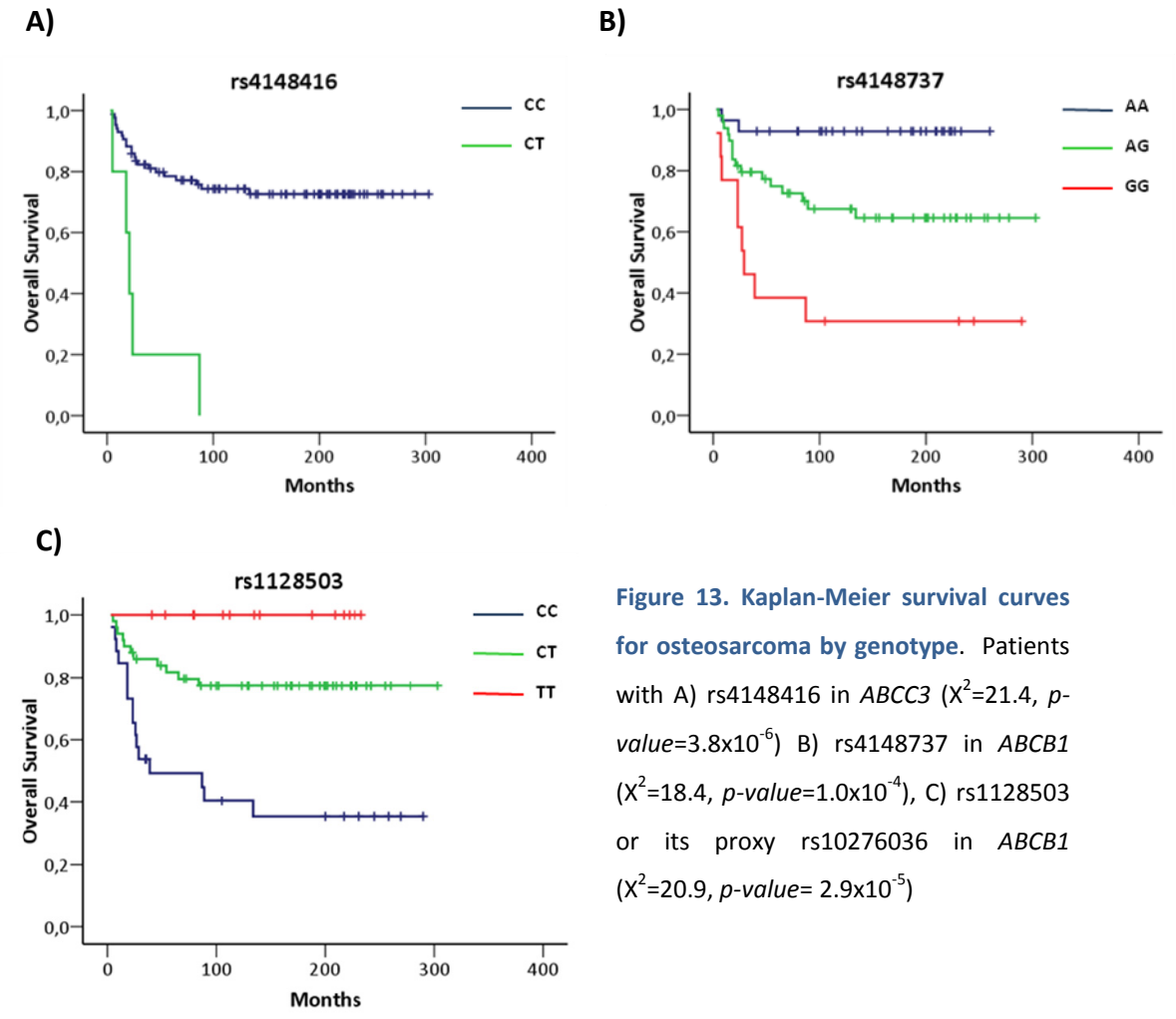


Figure 13. Kaplan-Meier survival curves for osteosarcoma by genotype. Patients with A) rs4148416 in *ABCC3* (X^2=21.4, *p-value*=3.8x10^{-6}) B) rs4148737 in *ABCB1* (X^2=18.4, *p-value*=1.0x10^{-4}), C) rs1128503 or its proxy rs10276036 in *ABCB1* (X^2=20.9, *p-value*= 2.9x10^{-5})

A combination of three SNPs located in *ABCB1* (rs1045642 / rs2032582/ rs1128503) was previously described as putatively functional in several studies (Sissung *et al*, 2008a), (Balcerczak *et al*, 2010), (Lal *et al*, 2008). The LD plot showing the linkage disequilibrium between these 3 variants is shown in Figure 14. We explored whether there is a haplotype formed by these three SNPs that is more significantly associated with survival in these patients. We observed two frequent haplotypes, one formed by the combination of the three common alleles (CGC) with a frequency of 0.47 and the other comprising the three rare alleles (TTT) with a frequency of 0.39. Considering CGC as reference, the TTT haplotype was associated with better survival (HR=0.31, 95%CI=0.15-0.62, *p-value*=0.001). The other haplotypes observed had lower frequency and were not statistically significantly associated with survival. Nevertheless, the estimated HR for the two haplotypes containing the rare T allele in rs1128503 (CGT and CTT) were consistent with it having a protective effect (HR=0.38 and 9.78x10^{-6}, respectively) in contrast with the other two

haplotypes containing the C wild-type allele (TGC and TTC; HR=1.26 and 1.95, respectively). These results suggested that the haplotypes were no more informative than the rs1128503 SNP alone.

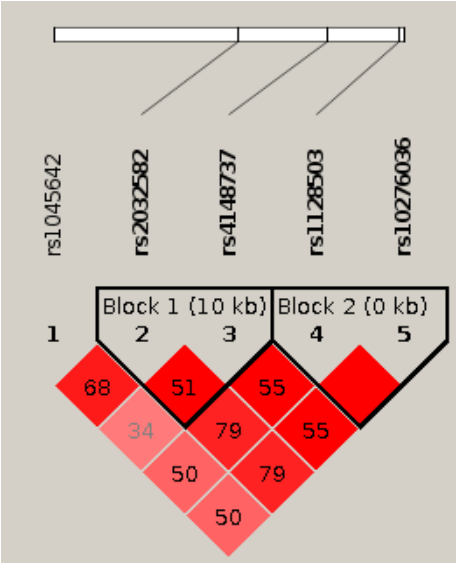


Figure 14. Linkage disequilibrium (LD) among the five studied variants in *ABCB1* gene. Pairwise r^2 measures calculated with the software package Haploview (version 4.1) are shown

Regarding the CNVs analyzed, genotype data for *GSTM1* were available for 98 patients, whereas data were available for 99 patients for the *GSTT1* CNV. The frequency of the homozygous gene deletion for *GSTM1* was 52% (51 patients) and 19% (19 patients) for *GSTT1*. There was no evidence that either of these two polymorphisms were associated with any of the clinical outcomes considered.

Results, part II

Pharmacogenetics of breast cancer treatment

2.1. Study III: Drug metabolism/transport gene variants related to docetaxel and doxorubicin treatment response in breast cancer patients

Taxanes and anthracyclines improve the outcome of early breast cancer, although the benefit is limited to a small percentage of patients. In this study, we used a candidate pathway approach analyzing SNPs in drug metabolism/transport genes to identify genetic variants related to doxorubicin response and docetaxel response in breast cancer patients. For this purpose, patients were randomized to receive doxorubicin (N=97) or docetaxel (N=85) as neoadyuvant therapy before surgery and genotyped for SNPs in the metabolism/transport pathway genes. We studied the association of the genotypes with tumour response, evaluated as clinical response and pathological response (RCB). A total of 186 out of 201 SNPs in 12 genes were successfully genotyped in 97 patients of the docetaxel arm. A total of 187 out of 204 SNPs in 13 genes were successfully genotyped in 97 patients of the doxorubicin cohort.

2.1.1. An ABCG2 polymorphism associates with docetaxel response

The SNP that better associated with the pathological response (RCB) was an intronic SNP in *ABCG2*, rs4148152 (p=0.0002). This SNP was associated with poor response, since the median RCB for AG patients was 4.21 compared with 2.26 of the AA wild type patients (Table 11).

Since this SNP is only 200 bp far from a coding SNP, rs2231137 (V12M) we genotyped our patients for this SNP using Kaspar genotyping assay and found they were in total linkage disequilibrium (r^2=1).

Table 11. Genes and SNPs associated with RCB

SNP	Allele	Median RCB	*P value*
Doxorubicin Arm			
ABCC2 rs717620	CC	3.15	
	CT	3.39	
	TT	4.32	
	T vs C		0.0040
Docetaxel Arm			
ABCG2 rs4148152	AA	2.26	
	AG	4.21	
	G vs A		0.0002

2.1.2. An ABCC2 polymorphism associates with doxorubicin response

The SNP that better associated with RCB was rs717620 in the 5'UTR of *ABCC2* gene (*p-value*=0.004).

Interestingly, the median RCB for patients carrying the TT polymorphic genotype of was 4.32 compared to 3.15 for CC patients (Table 11).

Since rs717620 is located in the promoter of the gene, therefore we analyzed the effect of the variant on *ABCC2* gene expression in normal liver tissues. Rs717620 was genotyped in DNA extracted from 50 frozen liver tissues by sequencing, as described in the Materials & Methods section.

We found that rs717620 was not significantly associated with gene expression changes (*p-value*=0.24).

This SNP has been previously reported to be associated with *ABCB1* upregulation (Ufer 2009, Hoffman 2007), thus we also explored the association between rs717620 and *ABCB1* gene expression and found that the rs717620 variant was associated with a significant difference in *ABCB1* mRNA levels. The median expression in normal liver tissues with the homozygous TT genotype was 2.1-fold higher than that of those with del-C heterozygous and CC homozygous genotypes (*p-value*=0.021; Figure 15)

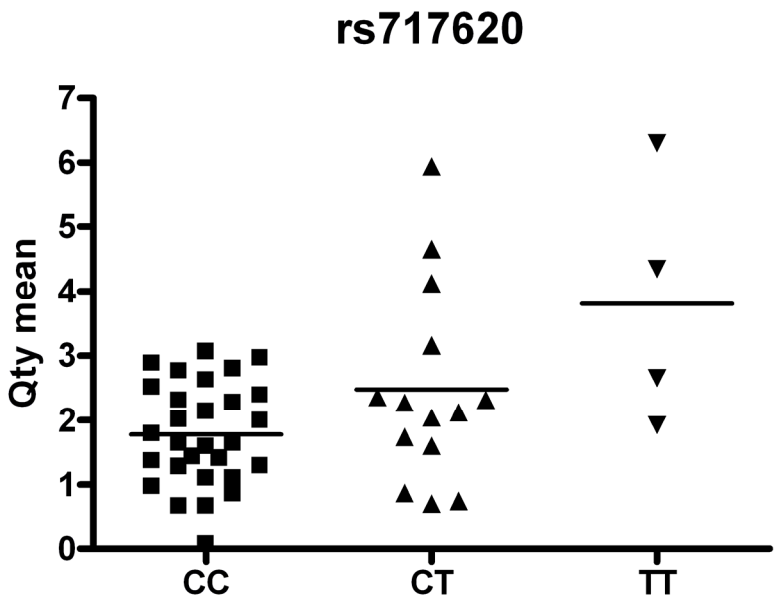


Figure 15. Effect of the rs717620 ABCC2 polymorphism on *ABCB1* gene expression in normal liver tissues

2.2. Study IV: capecitabine and drug metabolism pathway: hand-foot syndrome

Hand-foot syndrome (HFS) is one of the most relevant dose-limiting adverse effects of capecitabine, an oral prodrug of 5-fluouracil (5-FU) used in the standard treatment of breast cancer. The aim of the study is the identification of predictive markers for this specific ADR. We investigated the association between grade 3 HFS and genetic variation in the metabolic pathway of capecitabine using a candidate pathway approach. We genotyped a total of 13 polymorphisms in the capecitabine metabolism genes *CES2*, *CDD*, *TP*, *TYMS*, and *DPD* in 130 patients treated with capecitabine. We correlated these polymorphisms with susceptibility to grade 3 HFS.

The polymorphisms analyzed and the genotypic frequencies are shown in Table 12. The minor allele frequencies (MAFs) were between 0.06 and 0.42. There was no evidence of departure from Hardy-Weinberg equilibrium for any of them. Grade 3 HFS was observed in 41 (32%) of the 130 patients treated with capecitabine.

Table 12. Logistic regression analyses assessing associations of polymorphisms with grade 3 HFS

Genotype	Grade 0-2 HFS No (%)	Grade 3 HFS No (%)	Odds ratio	95% CI	*P-value*
CDD rs532545					
Dominant					
CC	37 (41.6)	11(26.8)	Referent		
CT/TT	52 (58.4)	30 (73.2)	2.28	0.95-5.44	0.057
Additive			**2.02**	**1.02-3.99**	**0.039**
CDD rs602950					
Dominant					
AA	37 (41.6)	13 (31.7)	Referent		
AG/GG	52 (58.4)	28 (68.3)	1.82	0.79-4.22	0.153
Additive			1.75	0.90-3.40	0.094
CDD rs2072671					
Dominant					
AA	36 (40.4)	13 (31.7)	Referent		
AC/CC	53 (59.6)	28 (68.3)	1.72	0.74-3.97	0.201
Additive			1.55	0.80-2.99	0.190
CES2 rs2241409					
Dominant					
CC	52(58.4)	26 (63.4)	Referent		
CT	37 (41.6)	15 (36.6)	0.71	0.31-1.60	0.403
Additive			0.78	0.40-1.52	0.456
CES2 rs11568314					
Co-dominant					
AA	78 (87.6)	37 (90.2)	Referent		
AT	11 (12.4)	4 (9.8)	0.74	0.20-2.71	0.648

Genotype	Grade 0-2 HFS No (%)	Grade 3 HFS No (%)	Odds ratio	95% CI	*P-value*
CES2 rs11568311					
Co-dominant					
GG	76 (85.4)	36 (87.8)	Referent		
GA	13 (14.6)	5 (12.2)	0.81	0.25-2.60	0.715
CES2 rs11075646					
Dominant					
CC	69 (77.5)	29 (70.7)	Referent		
CG/GG	20 (22.5)	12 (29.3)	1.43	0.59-3.47	0.434
Additive			1.32	0.62-2.85	0.475
TP rs470119					
Co-dominant					
GG	38 (43.2)	19 (46.3)	Referent		
GA/AA	50 (56.8)	22 (53.7)	0.91	0.41-2.00	0.811
Additive			0.94	0.52-1.70	0.846
TP rs131804					
Co-dominant					
AA	32 (36.0)	14 (34.1)	Referent		
AG/GG	57 (64.0)	27 (65.9)	1.09	0.48-2.46	0.842
Additive			1.03	0.57-1.85	0.927
TP rs11479					
Co-dominant					
CC	75 (86.2)	37 (90.2)	Referent		
CT	12 (13.8)	4 (9.8)	0.76	0.21-2.68	0.664
TYMS 3' UTR					
Dominant					
6bp/6bp	34 (38.2)	22(53.7)	Referent		
6bp/del	55 (61.8)	19 (46.3)	0.55	0.25-1.21	0.138
Additive			0.67	0.37-1.21	0.172
TYMS 5' UTR					
Dominant					
2R2R/ 2R3RC/3RC3RC	54 (62.1)	26 (63.4)	Referent		
2R3RG/3RC3RG/3RG3RG	33 (37.9)	15 (36.6)	1.10	0.49-2.49	0.821
Additive			1.02	0.53-1.93	0.960

(Table 12 continued)

2.2.1. Association of CDD rs532545 polymorphism with HFS

We found a significant association with HFS only for a polymorphism in the *CDD* gene. In particular, the polymorphic T allele of rs532545 was associated with higher incidence of grade 3 HFS: the estimated odds ratio (OR) was 2.02 (*p-value*= 0.039, 95%CI=1.02-3.99). No substantial changes were observed in this OR after adjustment for capecitabine dose, tumour type, age, and hepatic metastasis, while adjustment for dose reduction increased the significance of the

association. No evidence of association was found for the polymorphisms in the other genes considered (Table 2).

We found only one patient heterozygous for the *DPD* SNP rs3918290 and this patient experienced life-threatening toxicities (severe myelosupression and mucositis). This SNP was not included in the analyses. Lethal outcome or high toxicity has been reported after treatment with 5-FU or capecitabine.

2.2.2. Association of CDD rs532545 polymorphism with gene expression

The cytidine deaminase gene (*CDD*) is involved in the conversión of capecitabine to 5-FU.

Due to the SNP associated to HFS is located in the promoter of the *CDD* gene and had been described to affect transcription by Fitzgerald et al (Fitzgerald *et al*, 2006), we analyzed the relationship with *CDD* mRNA levels by quantitative real-time PCR. Since RNAs were not available for our patients, 89 lymphoblastoid cell lines from Caucasian healthy individuals were used for this purpose. After genotyping of rs532545 (MAF=0.29) in these cell lines we found that this SNP was not associated with a significant change in mRNA levels (*p-value*= 0.671).

Neither the other 2 SNPs in the *CDD* gene analyzed (rs602950, MAF=0.29 and rs2072671, MAF=0.36) were associated with gene expression (*p-values* of respectively 0.655 and 0.327 for rs602950 and rs2072671).

2.2.3. CDD gene promoter resequencing

We hypothesized that rs532545 might not be the causal SNP, but simply a marker SNP, so we searched by fine-mapping of the promoter for other variants that showed stronger association with HFS. We sequenced a 959 bp fragment at the 5' extreme of the *CDD* gene and found, apart from the 3 SNPs originally included in the study (rs602950, rs532545, rs2072671), 2 more common variants already annotated in the dbSNP database, rs3215400 and rs603412.

2.2.4. Association of rs3215400 polymorphism with HFS

We genotyped our series of 130 patients for these 2 variants and found a statistically significant association with HFS for rs3215400 (Table 13). In particular, carriers of at least one inserted C allele of rs3215400 had lower risk of developing grade 3 HFS (OR=0.37, *p-value*=0.020, 95%CI=0.16-0.86) compared to individuals homozygous for the deleted allele.

Table 13. Genotype distribution and logistic regression analyses assessing associations of rs3215400 and rs603412 with grades 3 HFS.

Genotype	Grade 0-2 HFS No (%)	Grade 3 HFS No (%)	Odds ratio	95% CI	*P-value*
CDD rs3215400 943insC					
Dominant					
- -	23 (25.8)	19 (46.3)	Referent		
-C/CC	66 (74.2)	22 (53.7)	0.37	0.16-0.86	0.020
Additive			0.51	0.27-0.95	0.028
CDD rs603412 -205C>G					
Dominant					
CC	28 (31.8)	12 (29.3)	Referent		
CG/GG	60 (68.2)	29 (70.7)	1.19	0.50-2.80	0.693
Additive			1.41	0.77-2.60	0.261

2.2.5. rs3215400 and rs532545 haplotype analysis

Further analysis was performed for this variant, comparing grade 0 *versus* 3 in order to better discriminate the HFS phenotype. Although the sample size was greatly reduced (N=96), the statistical significance was maintained (*p-value*=0.045). This variant is in linkage disequilibrium (LD) with the previously associated rs532545 (D'=0.92), and the LD block structure under the association interval is given in Figure 16.

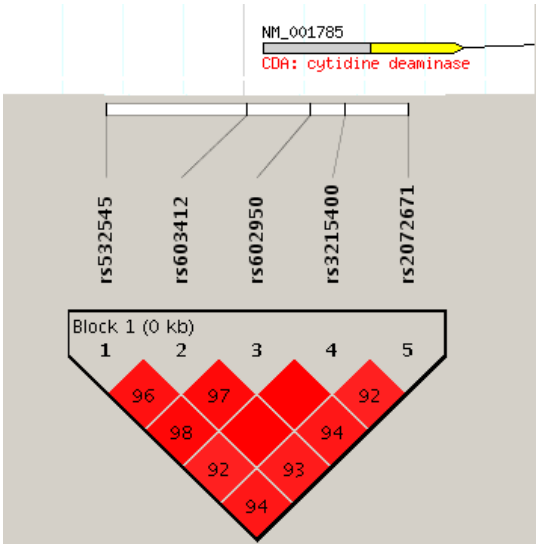


Figure 16. Linkage disequilibrium (LD) among the five variants studied at the CDD gene. Pairwise LD measures (D') calculated with the software package Haploview (version 4.1) are shown

To see if the effect we observed was due to a single variant or to a combination of the 2 associated polymorphisms, rs532545 and rs3215400, we also analyzed the effect of the

haplotypes on HFS. We found three frequent haplotypes; the most frequent was formed by the CG alleles of respectively rs3215400 and rs532545 with a frequency of 41% and was used as a reference. The –A haplotype had a frequency of 34% and an OR of 2.77 (*p-value*= 0.012, 95%CI=1.25-6.14) and the –G haplotype a frequency of 24% and an OR of 2.30 (*p-value*= 0.039, 95%CI=1.04-5.06).

Since the haplotypes that conferred an increased risk of HFS were only those that contained the deleted allele of the rs3215400 variant, these results suggest that the haplotypes were not more informative than the rs3215400 alone.

2.2.6. Association of rs3215400 with CDD gene expression

Using the same approach, we genotyped the 2 polymorphisms we found after promoter sequencing, rs3215400 (MAF=0.44) and rs603412 (MAF=0.44) in the 89 lymphoblastoid cell lines. Again, we investigated the correlation with gene expression and found that the rs3215400 variant was associated with a significant difference in *CDD* mRNA levels. The median expression in cell lines with the homozygous del-del genotype was 3.1-fold higher than that of those with del-C heterozygous and CC homozygous genotypes (*p-value*=0.004; Figure 17). The other *CDD* variant rs603412 was not significantly associated with gene expression (*p-value*= 0.4).

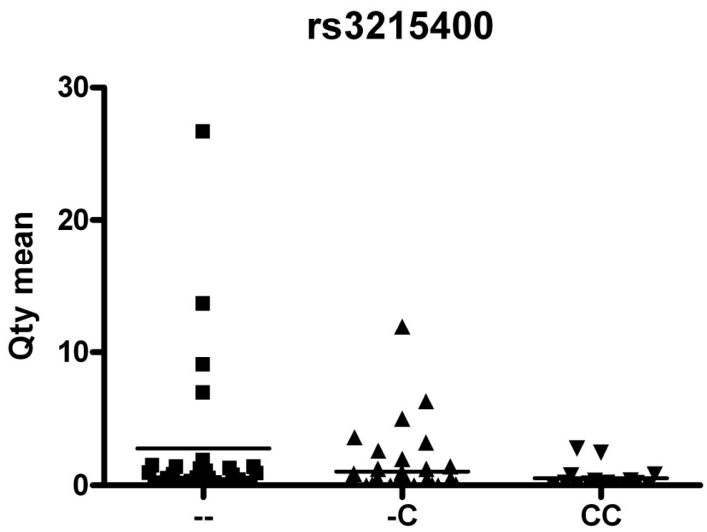


Figure 17. Effect of the rs3215400 CDD polymorphism on gene expression in Coriell lymphoblastoid cell lines.

2.2.7. In silico prediction for rs3215400

To elucidate whether the associated rs3215400 marker could be a putative functional variant, we carried out an *in silico* analysis using two different computer tools for the prediction of transcription factor binding sites, and we found that the deleted allele of rs3215400 abrogates a binding site for the transcription factor E2F (Figure 18).

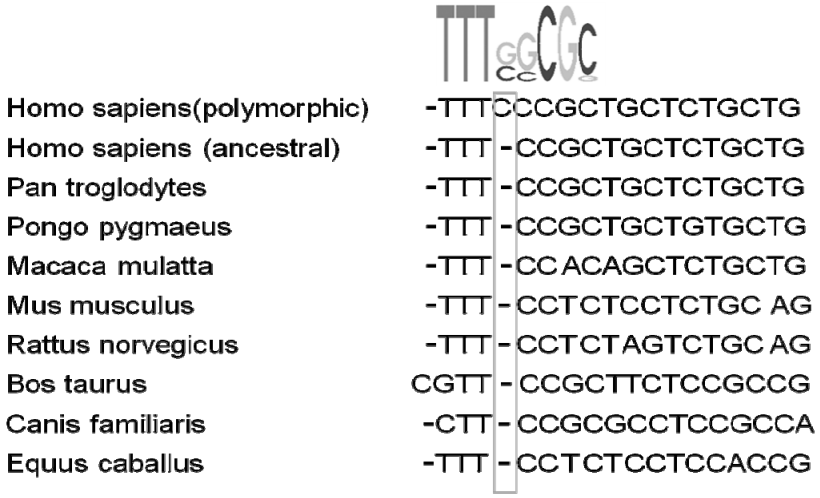


Figure 18. Transcription factor binding site prediction. The rs3215400 del allele abrogates the E2F binding site

2.2.8. Allelic imbalance analysis for rs3215400

Because of the global gene expression association and location in a putative transcriptional element, a more comprehensive analysis was performed. Specifically, we measured allele-specific expression (ASE) by SNaPshot in both genomic DNA and cDNA of the 43 cell lines heterozygous for rs3215400. As expected, the allelic ratio for genomic DNA was around 1, ranging from 0.95 to 1.6; by contrast, all samples analyzed displayed an increased allele ratio in cDNA compared to genomic DNA (Figure 19). In all cell lines analyzed, the ASE ratio for the deleted allele versus the C allele was higher than 3, with an average value of 5.7 and a range of 3.9-7.7 (standard deviation=0.83).

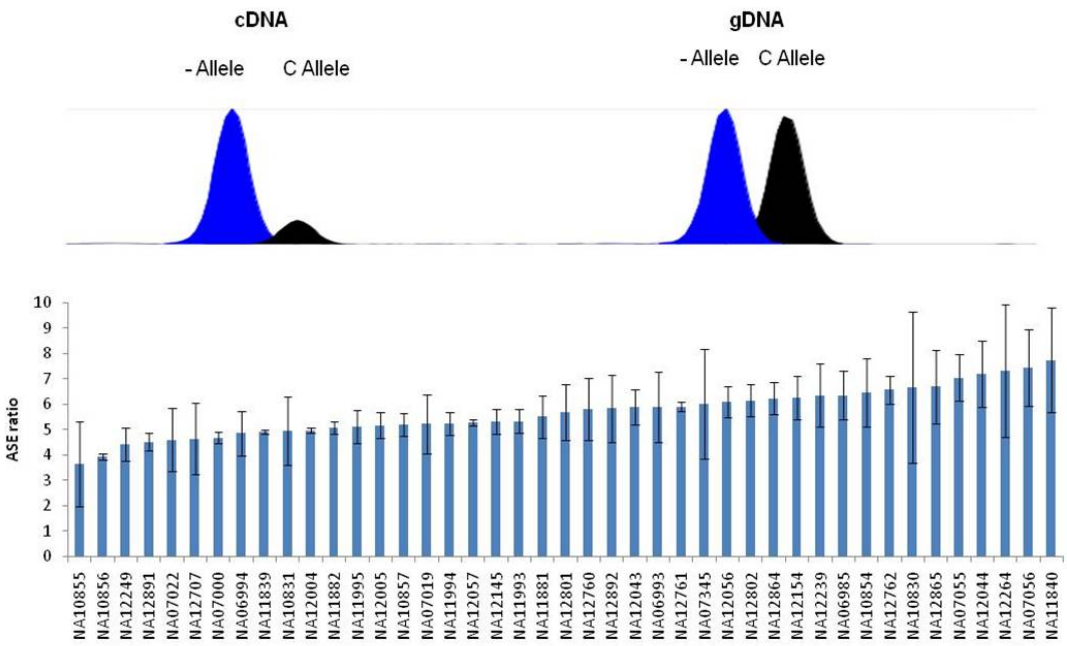


Figure 19. ASE analysis by SNaPshot in 43 Hapmap cell lines heterozygous for rs3215400. The upper part shows an example of the peaks obtained for the deleted allele (blue) and for the C allele (black) for cDNA (left) and for genomic DNA (gDNA; right). The ASE (lower part) ratio was calculated by normalizing the ratio between the peak areas of the two alleles in cDNA for the same ratio in the genomic DNA.

2.3. Study V: Genome-wide association study and capecitabine- induced hand-foot syndrome

Hand-foot syndrome (HFS) is one of the most relevant dose-limiting adverse effects of capecitabine but the *CDD* variant previously idenfied does not explain the whole interindividual variability found in patients treated with capecitabine, thus we performed a genome-wide analysis of these patients. In total we genotyped a total of 520871 SNPs in a total of 163 patients treated with this drug. In order to select patients having the most unequivocal phenotype possible we included patients that showed extreme discordant phenotype in the analysis (Crowley *et al*, 2009), (Turner *et al*, 2008), (Nebert, 2000a).

A total of 75 patients did not experience HFS during the treatment and were considered as grade 0, while 88 patients experienced grade 3 HFS.

For grade 0 patients, the total cumulative dose (mg/m^2 total) was calculated from the first cycle of treatment until the last treatment, while for grade 3 patients the cumulative dose was calculated from the first cycle to the first appearance of grade 3 HFS.

Of the clinical variable analyzed, sex and tumour type were associated with HFS and were included as covariable in the analysis. In particular, the HR for colorectal cancer *versus* breast cancer was 0.079, (95%CI=0.029-0.216, *p-value*=6.87x10^{-7}) while the HR for men *versus* women was 0.28 (95%CI=0.12-0.70, *p-value*=0.007).

2.3.1. Selection of the most significant variants

The distribution of *p-values* for associations, under an additive model, with HFS of SNPs across the genome is illustrated in the Manhattan plot (Figure 20). The most striking *p-value* (<10^{-9}) was for a SNP on chromosome 15 (Table 14). There are several limitations common to all GWAS including those related to response to treatment and prognosis (Manolio & Collins, 2009).

A key limitation is the potential for false-positive associations, particularly from single studies, but this can be overcome by testing results in independent series. We therefore selected a total of 16 genetic variants with *p-values* ≤10^{-5} for genotyping in the replication study (Table 14).

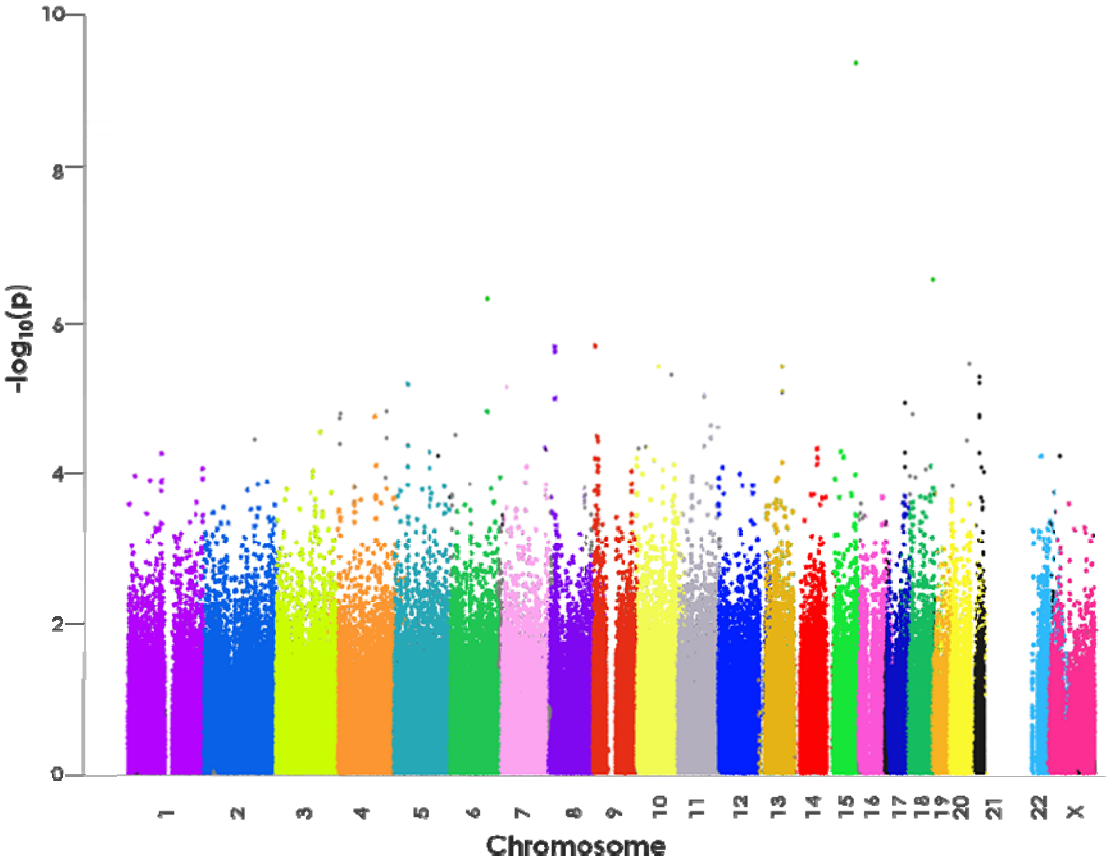


Figure 20. Manhattan plot of the *p-values*. Association with grade 3 HFS was assessed using Cox-regression analysis. The x-axis represents chromosomal location and the y axis shows *p-values* on a logarithmic scale

Table 14. SNPs with smallest *p-values* identified by GWAS analysis

		Unadjusted		*Adjusted for Sex and Tumour type*	
Chr*	SNP	HR (95%CI)	*P- value*	HR (95%CI)	*P- value*
15	***rs2619170***	4.99 (2.80-7.65)	$4.14x10^{-10}$	4.70 (2.70-7.49)	$2.92x10^{-9}$
18	***rs13381276***	3.24 (1.73-4.23)	$3.11x10^{-7}$	2.64 (1.62-4.03)	$2.97x10^{-5}$
6	***rs4962257***	2.63 (1.68-3.56)	$5.50x10^{-7}$	2.30 (1.53-3.27)	$2.00x10^{-5}$
6	***rs1121941***	2.63 (1.68-3.56)	$5.50x10^{-7}$	2.30 (1.53-3.27)	$2.00x10^{-5}$
9	***rs2755087***	2.57 (1.79-3.87)	$2.34x10^{-6}$	2.48 (1.63-3.60)	$8.78x10^{-6}$
8	***rs2237826***	2.19 (1.43-2.70)	$2.39x10^{-6}$	1.93 (1.40-2.59)	$3.14x10^{-5}$
8	***rs2237827***	2.18 (1.42-2.68)	$2.75x10^{-6}$	1.91 (1.38-2.57)	$4.28x10^{-5}$
20	***rs6093063***	2.23 (1.69-3.27)	$4.00x10^{-6}$	2.39 (1.67-3.23)	$2.50x10^{-7}$
10	***rs12221182***	2.97 (1.91-4.74)	$4.39x10^{-6}$	3.05 (1.91-4.72)	$1.60x10^{-6}$
13	***rs9573665***	2.57 (1.66-3.72)	$4.44x10^{-6}$	2.46 (1.62-3.60)	$9.85x10^{-6}$
10	***rs531790***	2.28 (1.46-2.97)	$5.74x10^{-6}$	1.99 (1.36-2.79)	$1.99x10^{-4}$
21	***rs7279195***	2.59 (1.63-3.71)	$6.12x10^{-6}$	2.33 (1.50-3.42)	$5.85x10^{-5}$
21	***rs7282914***	2.57 (1.62-3.68)	$7.33x10^{-6}$	2.31 (1.49-3.39)	$7.11x10^{-5}$
5	***rs730354***	2.13 (1.51-2.92)	$7.64x10^{-6}$	1.98 (1.41-2.73)	$4.76x10^{-5}$
7	***rs17337019***	2.81 (1.53-3.78)	$8.27x10^{-6}$	2.36 (1.48-3.65)	$2.13x10^{-4}$
13	***rs9593132***	3.61 (1.97-6.12)	$9.67x10^{-6}$	4.88 (2.39-8.14)	$1.58x10^{-6}$

*Chromosome

2.3.2. Genotyping of the most significant variants in the replication cohort

The 16 SNPs selected for replication were genotyped by Kaspar Genotyping Systems Assays in the 85 patients from the replication cohort described in the Materials & Methods and genotypes were assessed for association with grade 3 HFS.

Evidence of replication was observed for the SNP rs6093063 on chromosome 20 (HR=2.34, 95%CI=1.00-5.43, *p-value*=0.049), even after adjustment for tumour type (HR=2.71, 95%CI= 1.14-6.42, *p-value*=0.024) when the dominant model was considered.

SNP rs6093063 is intergenic, located 100Kb 5' upstream of the *CDH4* gene and 700 Kb downstream of the *MT-CO2* pseudogene. SNP imputation using both Hapmap Project and the 1,000 Genomes Project data is currently being done. Further genotyping and resequencing to fine map the region are currently being planned as the next steps towards identifying the causal SNP. Functional genomic studies will then be required.

DISCUSSION

Discussion, part I

Genetic variants associated with osteosarcoma treatment outcome and toxicities

1. Pharmacogenetics of osteosarcoma

The identification of molecular markers of response to treatment is essential for osteosarcoma patients, since there are few alternative treatments for those who relapse (Clark *et al*, 2008). The only known prognostic factors are the presence of metastasis, tumour necrosis after neoadjuvant chemotherapy, tumour location and tumour volume (Longhi *et al*, 2006). It is of great interest to explore the genetic variation in patients with osteosarcoma because the genetic profiles of each patient may also play a role in treatment response.

1.1. Study I: Common variants in DNA-repair genes are associated with response to cisplatin chemotherapy and ototoxicity in osteosarcoma patients

Cisplatin is one of the first-line chemotherapeutic agents used in the standard treatment of osteosarcoma. We found that carrying at least one G allele in *ERCC2* rs13181 conferred an estimated 5-fold risk of poor tumour response in osteosarcoma patients treated with this drug. This result was consistent with that observed for survival, with an associate almost 5-fold increased risk of relapse or death.

The association between this polymorphism and platinum response remains controversial. Several studies focused on oxaliplatin and colorectal cancer have reported associations between this SNP and poor clinical outcome (Park *et al*, 2001), (Ruzzo *et al*, 2008), (Stoehlmacher *et al*, 2004).

On the other hand, several studies have found no association between this polymorphism and cisplatin response in lung cancer patients (Giachino *et al*, 2007), (Isla *et al*, 2004), (Ryu *et al*, 2004); further studies are therefore warranted to confirm that *ERCC2* rs13181 is a predictor of cisplatin response.

After the publication of our findings (Caronia *et al*, 2009), Yin and colleagues (Yin *et al*) conducted a meta-analysis of relevant publications before 2010, based on a combined total of 1,787 cancer patients treated with oxaliplatin, and confirmed that *ERCC2* rs13181 was associated with poorer response and poorer survival. We also observed a non-statistically-significant trend of an increased risk of poor tumour response and reduced EFS associated with another variant in the *ERCC2* gene, the rs1799793 polymorphism.

Ruzzo and colleagues (Ruzzo *et al*, 2008) also studied this SNP and found that the association with platinum response seemed to be weaker than for rs13181, which is located 12 Kb downstream. In our patient series, we have observed a similar trend of a weaker

effect for rs1799793. This finding could be explained by the fact that these two SNPs are in linkage disequilibrium (D´=0.66).). Therefore, our results suggest that the *ERCC2* rs13181 polymorphism is the better predictive marker of the two for both clinical response and survival.

The mechanisms by which this polymorphism could affect platinum response are not clear. The *ERCC2* gene encodes a DNA helicase that is an essential component of the NER pathway. The impact of this polymorphism on DNA repair has been evaluated by functional studies with controversial results. Lunn and colleagues (Lunn *et al*, 2000) showed that carriers of the common T allele had a suboptimal DNA repair activity while Spitz et al reported that the polymorphic G variant was associated with lower DNA repair activity (Spitz *et al*, 2001). On the other hand, some reports didn't observe any evidence of association between this polymorphism and DNA repair activity (Duell *et al*, 2000), (Clarkson & Wood, 2005). The discrepancies observed between these studies could be due to the different assays used to measure DNA repair. Since they measure different parameters using assays such as X-ray induced chromatic aberrations and the host cell reactivation assay, some of them may not be appropriate for the evaluation of the effect of this polymorphism on DNA repair. Another possible explanation is that this polymorphism is not causal itself, but rather is in linkage disequilibrium with a functional polymorphism involved in cisplatin response. Rs13181 is a coding SNP (Lys751Gln) located in the last exon of *ERCC2*, close to the 3´UTR region. Variation in this region could affect the stability of mRNA or even the regulation of protein translation.

Our results also suggest that *XPC* may play a role in cisplatin efficacy. This gene encodes a protein of 940 amino acids that recognizes DNA damage, and *XPC* polymorphisms have previously been found to be associated with cancer risk (Hu *et al*, 2005), (Vogel *et al*, 2005). Additionally, the minor allele of *XPC* rs2228001 not only decreases risk of poor tumour response but may also be associated with higher risk of ototoxicity, although the limited number of patients included in our analysis limited our ability to reach definitive conclusions. To date, pharmacogenetic studies of cisplatin-induced ototoxicity have reported an association between polymorphisms in the drug metabolism gene *GSTP1* and ototoxicity in testicular cancer patients (Oldenburg *et al*, 2007), while Ross and colleagues identified functional genetic variants in thiopurine S-methyltransferase (*TPMT*) and catechol Omethyltransferase (*COMT*) which increased the risk of developing cisplatin-induced hearing loss (Ross *et al*, 2009).

This is the first study to report an association between *XPC* variation and hearing impairment, underlining the importance of DNA-repair genes not only in cisplatin response but also in ototoxicity.

A possible explanation for our observed association is that rs2228001 may reduce the activity of XPC and thus its DNA repair capacity. This decrease in DNA repair capacity could enhance apoptosis in response to platinum both in tumour cells, thereby increasing the cisplatin response, and in the normal outer hair cells of the organ of Corti, explaining the associated ototoxicity. Unfortunately, functional studies for this polymorphism have also yielded inconsistent results (Khan *et al*, 2000), (Zhu *et al*, 2007). Additional studies are therefore required to evaluate the role of variation in *XPC* in cisplatin response and ototoxicity.

Despite previously published studies reporting an association between *ERCC1* SNPs and platinum response (Zhou *et al*, 2004), (Viguier *et al*, 2005), there was no strong evidence of this in our study. We observed a tendency towards poor tumour response and reduced EFS in patients carrying the variant alleles of *ERCC1* rs3212986 and rs11615 but none of these reached statistical significance. Interestingly, the aforementioned meta-analysis by Yin and colleagues (Yin *et al*, 2011), also included the *ERCC1* rs11615 SNP and found that the effect of this SNP was statistically significant in Asian patients in particular. If this is the case, it could explain the result of our study that included only Caucasian patients.

The other variants in *XPA*, *ERCC5* and *ERCC4* analyzed were included in our study because they had previously been reported to be associated with cancer risk (Vogel *et al*, 2005), (Milne *et al*, 2006), (Carles *et al*, 2006) which was considered an indication of their potential implication in DNA repair efficiency. Despite this, we have found no evidence of an association with cisplatin response.

It should be highlighted that in the context of multidrug neoadyuvant therapy, the response to treatment and other patient outcomes may be the result of combinations of drugs, and we cannot rule out the possibility that the observed associations may be due to the respective polymorphisms influencing response to agents other than cisplatin.

However, there is currently no clear evidence in the literature of an association between NER and sensitivity to the other drugs used in combination with cisplatin in osteosarcoma. In fact, the NER pathway removes cisplatin-induced DNA adducts (Zamble *et al*, 1996) and, based on evidence published to date, doesn't seem to be involved in the repair of the damage induced by these other drugs.

Many pharmacogenetic studies of cisplatin have been conducted, the majority focusing on DNA repair genes and GSTs. Some studies have reported an association with the efficacy of, and toxicities to, cisplatin for *GSTT1* and *GSTM1* copy number, as well as GSTP1 polymorphisms (Funke *et al*, 2010), (Khrunin *et al*, 2009), (Moyer *et al*, 2010). Specifically, *GSTT1* and *GSTM1* CNVs were associated with clinical outcome in a study of 80 osteosarcoma patients treated with platinum agents (Salinas-Souza *et al*, 2010). We also studied these polymorphisms in our patients (see Study II) but did not replicate these associations. New insights into the genetic basis of interindividual differences in cisplatin response have come from GWAS: recently Wu and colleagues (Wu *et al*, 2011) reported an association between a SNP in chemokine-like receptor 1 (*CMKLR1*) and poor overall survival for platinum-treated patients with non-small cell lung cancer. These findings require further exploration, but they demonstrate the utility of GWAS in identifying genes previously not known to be related to drug response. Other studies are being performed using a GWAS approach in lymphoblastoid cell lines, assessing associations between genotypes and sensitivity to cisplatin (Huang *et al*, 2007), (Wheeler *et al*, 2010). While emerging results will need to be clinically validated, this novel approach offers an interesting strategy in the search for novel pharmacogenetic variants. Currently, in our laboratory we are performing a GWAS analysis of ototoxicity in our series of osteosarcoma patients treated with cisplatin.

In conclusion, to our knowledge, this is the first study showing the involvement of SNPs in DNA-repair genes in the response of osteosarcoma patients to chemotherapy. We found that polymorphisms in the *ERCC2* gene, specifically *ERCC2* rs13181, may be informative markers for the prediction the response to cisplatin treatment and clinical outcome. Furthermore, for the first time we have identified a possible role of *XPC* in cisplatin response. However, further studies with larger numbers of patients are required to confirm our findings. Functional analyses are also required to elucidate the role of these associated SNPs in DNA repair activity.

1.2. Study II: Common variants in ABCB1 and ABCC3 are associated with clinical outcome in osteosarcoma patients

This study assessed 346 SNPs and 2 CNVs in 24 key genes involved in pathways related to platinum, doxorubicin, methotrexate, vincristine, and cyclophosphamide, and is therefore the most comprehensive pharmacogenetic study conducted to date in osteosarcoma. The use of large-scale genotyping methods to screen multiple drug-related genetic pathways

has enabled us to identify four SNPs, one in *ABCC3* and three in *ABCB1*, which are strongly associated with overall survival and might therefore be useful prognostic markers in these patients.

The majority of pharmacogenetic studies carried out to date have been biased in their selection of variants towards polymorphisms described as functional in previously published studies. However, given the complexity of the problem, and the lack of understanding of both genetic effects and the regulation of chemotherapy action, these clearly functional polymorphisms likely explain only a portion of the observed phenotypic variability in treatment outcomes. In our study, we have assessed all genes known to be involved in the metabolism and transport of the drugs used in osteosarcoma treatment. In order to avoid the aforementioned bias in variant selection, we included not only previously described and other potentially functional polymorphisms, but also tagSNPs. This strategy ensures a more comprehensive evaluation of the contribution of common variation in these genes, therefore maximizing the potential to detect novel markers that could play a role in the interindividual differences observed in the clinical outcomes of osteosarcoma patients.

ABCC3 is a member of the multidrug resistance protein (MRP) family and is expressed in liver, gallbladder, kidney, and gut (Borst *et al*, 2000), (Rost *et al*, 2001). The main substrates of ABCC3 are bile salts (Hirohashi *et al*, 2000), but this protein also transports anticancer drugs, such as methotrexate (Zeng *et al*, 2000). Less clearly established ABCC3 substrates include vincristine, doxorubicin and cisplatin (Zeng *et al*, 2000), (Young *et al*, 2001). The expression of ABCC3 mRNA has been related to drug resistance but to date few published studies have assessed the direct implication of polymorphisms in *ABCC3*. Only non-synonymous coding and promoter SNPs have been investigated as potentially functional variants (Kobayashi *et al*, 2008), (Lang *et al*, 2004), but, to our knowledge, none have been found to be associated with survival after treatment in cancer patients. In the present study we found that SNP rs4148416 in this gene was associated with an estimated 8-fold increased risk of death and this is the first evidence of its clinical relevance. This SNP leads to a synonymous change (G1013G) on exon 22.

The other SNPs we found associated with osteosarcoma survival are located in the *ABCB1* gene. This gene is well-known and encodes a P-glycoprotein, an ATP-driven efflux pump that is overexpressed in many tumours and confers multidrug resistance (Juranka *et al*, 1989). Of the drugs administered to these patients, both doxorubicin and vincristine are transported by this pump (Cascorbi & Haenisch, 2009).. There are three variants that have

been studied in detail: 2677G>T/A (rs2032582), 3435C>T (rs1045642) and 1236C>T (rs1128503). The first is a non-synonymous change, while the other two are synonymous. These three SNPs have been studied both individually and as a haplotype, but results have been inconsistent (Kimchi-Sarfaty *et al*, 2007), (Sissung *et al*, 2008a), (Lal *et al*, 2008), (Morita *et al*, 2003). The rare allele of rs1045642 has been reported to be associated with reduced P-glycoprotein activity, both alone and in combination with the rare alleles of rs2032582 and rs1128503 (Kimchi-Sarfaty *et al*, 2007), (Hoffmeyer *et al*, 2000). These latter two variants have also been reported by different studies to have independent functional effects (Sakaeda, 2005), (Jamroziak *et al*, 2009), (Hoffmeyer *et al*, 2000). However, it is still not clear which is/are the functional variant/s in this gene.

In our study, of these three variants, only 1236T>C (rs1128503) was associated with survival after correction for multiple testing. This SNP leads to a synonymous change at residue 412 of the protein and is well-described but there is no clear consensus on its functional significance (Leschziner *et al*, 2007). Some studies have observed increased drug response in the presence of the T allele (Mathijssen *et al*, 2003), (Zhang *et al*, 2008) while others found the opposite (Schaich *et al*, 2009), or no association at all (Estrela Rde *et al*, 2009). In our study, the T allele was associated with better survival. To determine whether the observed association was due to this single variant or to a combination of these three polymorphisms, we also assessed associations with overall survival for the haplotypes they form. The results were consistent with a single main effect for rs1128503. Consistent results were reported by Balcerczak and colleagues for colorectal cancer patients (Balcerczak *et al*, 2010).

We found another SNP, rs10276036, associated with survival that was in complete LD with the rs1128503. It is located in intron 9 and has been linked with reduced area under the curve (AUC) of SN-38, the active metabolite of CPT-11, (Innocenti *et al*, 2009); however its functional significance is unknown. The observed association could be explained by the correlation with rs1128503, rather than by rs10276036 itself. In addition, we identified a polymorphism located in intron 17 (rs4148737) that was also strongly associated with survival. This SNP was in low LD with the other two SNPs, suggesting that the association observed could be due to an independent effect.

We hypothesize that genetic variation in these two transporter genes could have an effect on the efflux of the drugs used in the treatment of osteosarcoma, thus impairing the response to treatment and therefore the overall survival. Although we did not observe

evidence of association between these variants and tumour response, this could be explained by the fact that tumour response is evaluated after the administration of neoadjuvant therapy and at this point patients have been treated exclusively with methotrexate, cisplatin, and doxorubicin. Therefore, the tumour necrosis data does not evaluate the effect of vincristine and cyclophosphamide which are both given after surgery. Since ABCB1 is known to transport vincristine, ABCC3 could also be involved in this process (Young *et al*, 2001), (Huang *et al*, 2006), and the transport mechanisms for cyclophosphamide are still unknown, we postulate that genetic variation in these transporters could play a role in the effectiveness of the whole multidrug treatment, neoadjuvant and adjuvant, and in this way influence the overall survival.

In conclusion, this study identified four SNPs in two drug transporter genes that are associated with overall survival in osteosarcoma patients. After validation in large and well-defined sets of patients to confirm the associations, these variants could be useful as prognostic markers in these patients.

Furthermore, the approach used in this study, integrating multiple drug pathways and studying a large number of polymorphisms, may be extended to future pharmacogenetic studies to provide a more comprehensive interrogation of common genetic factors in candidate pathways that influence drug efficacy and toxicity. The applicability of high-throughput genotyping chips that enable the simultaneous analysis of multiple polymorphisms will facilitate research in this field.

Discussion, part II

Genetic variants associated with breast cancer treatment outcome and toxicities

2. Pharmacogenetics of breast cancer

Breast cancer survival has increased by approximately 5% in recent years in Western Europe. The maintenance of this survival gain in the future requires further improvements in disease management that include the optimal use of the different therapy strategies (schedule and dose administration), based on tailored chemotherapy according to the genetic profile of each patient. Therefore, the identification of polymorphisms that determine interindividual variability in drug tolerance is needed.

In this thesis, we studied the role of genetic variants in breast cancer treatment response and adverse drug reactions.

2.1. Study III: Genetic variants related to docetaxel and doxorubicin response in breast cancer patients

Neoadjuvant chemotherapy based on anthracycline and taxanes is increasingly being used to improve the outcomes of patients with large and locally advanced breast tumors. In this study, we evaluated the role of variants in genes involved in the metabolism and transport of docetaxel and/or doxorubicin on treatment response in breast cancer patients treated with each of these drugs in monotherapy.

Several pharmacogenetic studies of docetaxel have been performed, some of them considering polymorphisms in the *ABCB1* transporter gene and their associations with response and adverse effects. Sissung et al studied 23 patients treated with docetaxel alone and identified an association of *ABCB1* SNPs with neuropathy, neutropenia, and survival (Sissung *et al*, 2008a). They also studied other genes from the cytochrome 450 family involved in oxidative metabolism of the drug, and found an association of allele *3 of the *CYP1B1* gene with survival (Sissung *et al*, 2008b). Given the small sample size, these results should be treated with caution and validated in other larger series of patients. More recently, in a study by Deeken et al, a high-throughput platform (Affymetrix DMET) (Deeken *et al*, 2010) was used to simultaneously evaluate variation in genes involved in the metabolism, transport, and excretion of drugs in general, and several SNPs associated with response to, and toxicity ofm docetaxel were identified. Surprisingly, none of the reported associations involved genes previously recognized as being involved in the metabolism, transport, and activity of this drug. Since the study was also conducted on a very limited number of patients (25 men with prostate cancer treated with docetaxel alone), the results are far from conclusive and require further validation.

Several pharmacogenetic studies of doxorubicin have also been performed. In the most recently published, Bray and colleagues (Bray *et al*, 2010) analyzed polymorphic variants in the *ABCB1, SLC22A16, CYP2B6, CYP2C9, CYP2C19,* and *CYP3A5* genes in breast cancer patients treated with doxorubicin and cyclophosphamide; they identified SNPs in *ABCB1, SLC22A16*, and *CYP2B6* associated with response to treatment. This study has several limitations; not all genes involved in the metabolism of doxorubicin were explored and the response evaluated was not exclusively to doxorubicin but is rather a joint response to doxorubicin and cyclophosphamide treatment.

Another study reported an association between a SNP in *ABCC2* and doxorubicin-induced cardiotoxicity (Wojnowski *et al*, 2005), highlighting the relevance of this gene in doxorubicin transport.

In our study we found that the coding SNP rs2231137, located in the transporter gene *ABCG2* was associated with docetaxel efficacy.

ABCG2, also known as breast cancer resistance protein (BCRP), is a member of the ATP-binding cassette transporter super family and reduces exposure to many drugs.

ABCG2 is expressed in placenta, small intestine, liver and in breast ducts and lobules. Many drugs used in oncology are transported by ABCG2, and increased expression of this protein has been associated with poor response to chemotherapy (Robey *et al*, 2009).

SNPs in *ABCG2* have been studied because of their relevance to drug response (Tamura *et al*, 2007), (Morisaki *et al*, 2005). The coding variant G34A V12M (rs2231137) was previously observed to affect response to anticancer drugs (Mizuarai *et al*, 2004) and to increase ABCG2 transport activity (Ishikawa *et al*, 2005). We therefore postulate that the polymorphic allele could increase the transport activity in these patients, thus rendering them resistant to therapy. Consistent results were reported by Wang and colleagues in acute laeukemia patients treated with ABCG2-transported drugs such as mitoxantrone and daunorubicin (Wang *et al*, 2011) and by Hu and colleagues in patients with large B-cell lymphoma (Hu *et al*, 2007).

We also found that the promoter SNP rs717620 (-24C>T) in *ABCC2* was associated with doxorubicin efficacy.

This variant has been found to be associated with decreased expression of *ABCC2* (Haenisch *et al*, 2007) and with poor response to antiepileptic pharmacotherapy (Ufer *et al*, 2009), and to platinum (Han *et al*, 2011).

Given that the minor T allele has been associated with lower expression, we anticipated that we would observe a better response to pharmacotherapy in carriers of this allele. Surprisingly, our study, as well as that by Ufer and colleagues, indicates that the minor allele as associated with poor response. Ufer and colleagues suggested a converse upregulation of the *ABCB1* gene product as a possible explanation for this finding, based on their finding that the highest *ABCB1* mRNA expression was observed for *ABCC2*-24TT patients (Ufer *et al*, 2009). The upregulation of the ABCB1 pump had already been reported in ABCC2 knockout rats, and is believed to be a compensatory effect for the absence of ABCC2 (Hoffmann & Loscher, 2007). We evaluated the expression of *ABCB1* in relation to *ABCC2* rs717620 genotypes in normal liver tissues and found that the variant T allele was associated with increased levels of *ABCB1* mRNA, thus confirming the findings of Ufer and colleagues. The upregulation of the multidrug resistance pump ABCB1 therefore appears to be the mechanism by which rs717620 affects doxorubicin response.

2.2. Study IV: variants in genes related to capecitabine metabolism and the development of hand-foot syndrome

The introduction of capecitabine as a treatment for metastatic breast cancer has resulted in several benefits in the management of these patients. These are related to its effects on disease control, its favourable safety profile and its convenient oral dosing schedule. However, many patients experience adverse drug reactions; specifically, hand-foot syndrome is the one most frequently observed. In this study, we aimed to better elucidate the genetic mechanisms related to the appearance of this side effect. We followed two different strategies to identify novel genomic regions associated wth HFS: a candidate gene approach focused principally on genes already known to be involved in capecitabine metabolism, and a genome-wide approach.

Using the candidate-pathway approach we found an association between the rs3215400 polymorphism in the *CDD* gene and the development of grade 3 HFS. The *CDD* gene encodes an enzyme involved in the pyrimidine salvage pathway and irreversibly catalyses the hydrolytic deamination of cytidine and deoxycytidine to their corresponding uridine derivatives (Fitzgerald *et al*, 2006). In addition, CDD plays an essential role in the metabolism of a number o f antitumour cytosine nucleoside analogues, leading to their pharmacological activation to 5-FU.

To date, several studies have assessed the relationship between polymorphisms in the *CDD* gene and sensitivity to cytosine nucleoside analogues and related toxicities. The G208A

polymorphism has been reported to be associated with arabinoside-C sensitivity (Yue *et al*, 2003) and gemcitabine-related toxicities in the Japanese population (Yonemori *et al*, 2005), but it was not included in our study since it is very rare in Caucasian populations (Gilbert *et al*, 2006), (Fukunaga *et al*, 2004). The 79A>C variant (rs2072671), which we did include in our study, has been reported to be related to gemcitabine sensitivity, but there is no clear evidence of a functional role or association with toxicities (Yue *et al*, 2003). Fitzgerald et al (Fitzgerald *et al*, 2006) reported a decrease in *CDD* expression associated with a haplotype formed by rs602950 and rs532545; but since this haplotype is very rare (less that 1%) in our series of patients as well as in cell lines, it cannot account for the differences in expression observed. To our knowledge, only Ribelles et al (Ribelles *et al*, 2008) have evaluated the *CDD* rs3215400 polymorphism in relation to HFS, but no significant association was found. Additional functional analyses performed in cell lines support our finding by demonstrating that the association observed in patients probably reflects the effect of this regulatory variant on *CDD* expression. Of the five common variants we analyzed at the *CDD* locus, rs3215400 was the one most strongly associated with HFS. We hypothesized, based on *in silico* analysis, that the presence of a C allele at position 943 of *CDD* is critical for transcriptional suppression of the gene, resulting in reduced protein production, and that such suppression is mediated through E2F binding. This functional explanation is consistent with our observation that the deleted allele is associated with an increase of global *CDD* gene expression. We also observed consistent results in terms of allele-specific expression, with the deleted allele expressing 3- to 7-fold higher mRNA levels than the inserted allele. Since these functional experiments performed in lymphoblastoid cell lines demonstrate notable differences in the transcriptional activities of the deleted and C alleles, we infer that this could also happen in patients in vivo, and therefore that the rs3215400 variant could be causal.

In agreement with our functional data, it has been reported that the CDD extensive metabolizer phenotype could be responsible for the severe toxicities to capecitabine (Ciccolini *et al*, 2010). Indeed, CDD activity has been demonstrated to be directly correlated with severe toxicities after capecitabine or gemcitabine administration (Mercier *et al*, 2009) (Ciccolini *et al*, 2010).

On the basis of our findings, we propose a model in which the absence of an E2F site within the *CDD* promoter enhances *CDD* transcription (Figure 21). This could also take place in normal tissues. In particular, cell cytotoxicity could be accentuated by the elevated proliferation rate observed in the skin of the palm and sole and also by the increased

expression of TP observed in these tissues (Milano *et al*, 2008) rendering them more sensitive to the cytotoxic effects of 5-FU. This model may explain at least part of the phenotypic differences observed among treated patients.

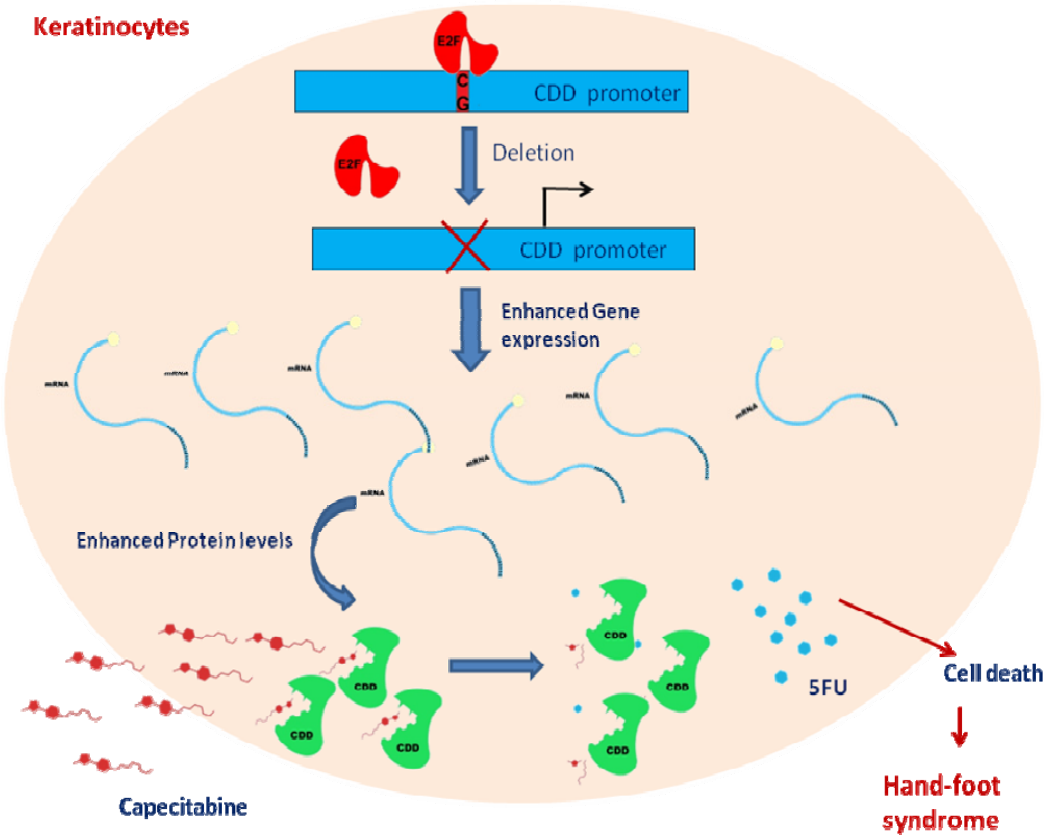


Figure 21. Proposed model to explain the direct effect of CDD rs3215400 on the development of hand-foot syndrome. The deletion of a C allele abrogates a binding site for of the E2F transcription function, inducing gene expression. The resulting enhanced expression leads to an increased conversion of capecitabine to 5-FU, which in turn induces to cell death in normal tissue, thus causing hand-foot syndrome.

Although our finding requires replication through more extensive and independent series of patients, our results provide evidence that rs3215400 in the *CDD* gene is a risk factor for HFS.

This pharmacogenetic study provides new insight into the clinical toxicity associated with capecitabine treatment.

2.3. Study V: genetic variants identified by GWAS related with hand-foot syndrome

In the previous study presented in this thesis we found a genetic variant in the capecitabine metabolism gene *CDD* related to HFS, but a large portion of the interindividual phenotypic variability still remains unexplained. The purpose of this study was both to identify predictive markers for HFS and to explore mechanisms that might explain this drug-related adverse event in patients exposed to capecitabine. Using a genome-wide interrogation of genetic variation, we discovered a new locus involved in the appearance of HFS in capecitabine-treated breast and colorectal cancer patients, which is not involved in pharmacokinetics and pharmacodynamics of this drug (that is, it would never have been identified through a candidate-gene/pathway approach).

The mechanisms that lead to hand-foot syndrome are still unclear. To date, HFS has been linked to the increased expression of TP and DPD metabolizing enzymes in the skin of the palms of the hands and soles of the feet (Milano *et al*, 2008), but additional research into the pathogenesis of HFS is needed. The genome-wide strategy offers the potential to identify new genes involved in the appearance of this side effect, but there are no published GWAS studies of HFS. A crucial point in this type of association analysis is to identify and accurately and consistently classify the phenotype into ordinal groups, in this case grade of severity. This is especially important when patients come from different hospitals and, in the case of HFS, when the phenotype assignation depends on the subjectivity of the clinicians and patients in each of these hospitals. However, standard methods of analysis such as Cox regression are not easily adapted to outcomes measured on an ordinal scale. One way to overcome this problem is to examine the extreme ends of the distribution(Nebert, 2000a); we adopted this approach for our analysis by considering grade 3 HFS (the most severe grade observed) as the outcome of interest. This seems to be a good approach to increase the power to detect associations in case of pharmacogenetic studies, which are often limited by the difficulty of collect a series of patients that have been homogeneously treated. Another strength of our study is that the phenotype (appearance of grade 3 HFS) was analyzed considering its dependency or cumulative dose of capecitabine, rather than just evaluating the presence or lack of HFS at the time of clinical data collection. This approach avoids biases resulting from the inclusion of patients classified as grade 0 that could be at risk of experiencing grade 3 HFS but did not receive a sufficient cumulative dose of capecitabine to cause the side-effect; these patients were censored at the total cumulative dose received.

Our GWAS analysis identified 16 variants potentially associated with the appearance of grade 3 HFS with a p-value less than 10^{-5}. Only the association between rs6093063 and HFS was replicated in an independent series of patients. This SNP was associated with an increased risk of developing grade 3 HFS with an adjusted *p-value* in the discovery series of $2.50x10^{-7}$, close to the genome-wide significance threshold of 10^{-7}. This association was replicated in an independent cohort of 85 patients.

SNP rs6093063 is intergenic, located 100Kb 5' upstream of the *CDH4* gene and 700 Kb downstream of the pseudogene *MT-CO2*. We hypothesize that this SNP could be located in a regulatory region of *CDH4* because this is the closest gene. CDH4 encodes R-cadherin. Cadherins mediate Ca^{2+}-dependent cell-cell adhesion which plays an important role in the formation and maintenance of tissue integrity (Kitagawa *et al*, 2000).

Specifically, CDH4 appears to play an important role in maintaining tissue architecture and cell polarity. CDH4 has been reported to be repressed by promoter methylation in human gastrointestinal tumours and to be downregulated in breast cancer(Miotto *et al*, 2004), (Agiostratidou *et al*, 2009).

Since HFS is characterized by desquamation, blister and plaque formation, a possible explanation for the observed SNP association could be that an alteration of CDH4 cadherin activity enhances the disruption of tissue integrity. Considering that the SNP array used in our GWAS study was designed using surrogated markers chosen to capture LD structure, rather than being based on any functional rationale. Therefore an evaluation of all common SNPs at this locus, using data from both Hapmap Project and the 1,000 Genomes Project, will be essential to decipher the disequilibrium structure and identify new variants that might be functional. This list of variants should then be refined by genotyping them in our patient series to ensure that they are independently associated with HFS, and then finally the functional role in HFS of those potentially causal variants remaining will require assessment using several approaches.

In summary, this GWAS identified a novel genomic region that implicated in HFS appearance in capecitabine treated patients. This association may provide valuable biological insight into the development of this adverse drug reaction, adding to what is already know about the pharmacokinetics and pharmacodynamics the drug. However, the mechanism by which variation at this locus increases HFS risk has not been established. Further studies are needed to elucidate the genetic basis of this association and its functional impact on HFS appearance.

CONCLUSIONS

1. The pharmacogenetic studies carried out in this thesis have led to the identification of genetic variants associated with treatment response and the development of adverse effects in osteosarcoma and breast cancer patients. These variants can be used as predictive biomarkers and represent a great advance for these patients towards what has been termed *personalized medicine*.

2. The use of high-throughput genotyping technologies has enabled the simultaneous assessment of multiple polymorphisms which has greatly facilitated research in this field.

3. Throughout this doctoral thesis, we have employed two different but complementary approaches. One the one hand, we have carried out *candidate gene* studies, assessing the set of genes involved in the pharmacokinetics and pharmacodynamics of the therapeutic agents considered. On the other hand, we also adopted a hypothesis-free strategy under which we assessed common genetic variation across the entire genome, and this enabled us to identify new candidate genes. The application of these two approaches in combination has led to the identification of new pharmacogenetic variants, thereby contributing to the field a more comprehensive outlook with regard to genetic variants that may influence drug efficacy and toxicity.

4. We have identified two coding genetic variants, rs13181 in the gene *ERCC2* and rs2228001 in *XPC*, both from the nucleotide excision repair pathway, that appear to be involved in response to cisplatin and which could play a role in the development of ototoxicity in osteosarcoma patients.

5. We have identified four polymorphisms (two synonymous coding and two intronic) located in two ATP binding cassette (ABC) transporters, *ABCC3* and *ABCB1*, that are associated with the efficacy of standard treatment in osteosarcoma patients. In terms of treatment for breast cancer, we have also identified a coding SNP in *ABCG2* associated with the efficacy of docetaxel and a

SNP in the promoter region of *ABCC2* associated with response to doxorubicine. These results suggest that common genetic variation in these ABC transporter genes may play an important role in treatment response, and would therefore be excellent candidates in the study of other tumours that are treated with agents that are transported by these proteins.

6. We have identified a SNP (rs3215400) in the promoter region of the *CDD* gene that is associated with an increased risk of hand-foot syndrome in breast and colorectal cancer patients treated with capecitabine. Furthermore, we have observed that this SNP affects the expression of CDD, and could therefore cause an increased conversion of capecitabine into its active form, 5-FU, resulting in increased toxicity.

7. In order to implement the variants identified in this doctoral thesis as biomarkers in clinical practice, they should first be validated in large independent series of patients. This should be done via the creation of large consortia that integrate research and clinical practice and thereby enable the application of these predictors to improve patient outcomes.

CONCLUSIONES

1. Los estudios farmacogenéticos que se han llevado a cabo en esta tesis doctoral nos han permitido identificar variantes genéticas asociadas con la respuesta al tratamiento y a la aparición de efectos adversos tanto en pacientes con osteosarcoma como con cáncer de mama. Estas variantes podrán ser utilizados como biomarcadores predictivos y son un gran avance hacia la denominada medicina personalizada en estos pacientes.

2. La utilización de tecnologías de genotipado de alto rendimiento nos ha permitido llevar a cabo el análisis simultáneo de múltiples polimorfismos facilitando enormemente la investigación en este campo.

3. A lo largo de esta tesis doctoral hemos empleado dos aproximaciones diferentes pero complementarias. Por un lado hemos llevado a cabo estudios en *genes candidatos* analizando el conjunto de genes que intervienen en la farmacocinética y farmacodinámica de los fármacos estudiados. Por otro lado, hemos empleado una estrategia *sin hipótesis previa* donde se ha analizado la variación genética del genoma completo y que ha permitido la identificación de nuevos genes candidatos. La combinación de estas dos aproximaciones ha permitido la identificación de nuevas variantes farmacogenéticas proporcionado una visión más extensa y global de los factores genéticos que puedan influir en la eficacia y toxicidad de los fármacos.

4. Hemos identificado dos variantes genéticas codificantes, rs13181 y rs2228001 en los genes *ERCC2* y *XPC* de la ruta de reparación del ADN por escisión de nucleótido que parecen estar involucradas en la respuesta a cisplatino y podrían jugar un papel en la aparición de ototoxicidad en pacientes con osteosarcoma.

5. Hemos identificado cuatro polimorfismos (dos codificantes sinónimos y dos intronicos) localizados en dos transportadores de tipo ABC (ATP binding cassettes) *ABCC3* y *ABCB1* asociados con la eficacia al tratamiento *estandar* en pacientes de osteosarcoma. Por otro lado, hemos identificado otros dos SNPs (un codificante y el otro localizado en el promotor del gen) en los genes *ABCG2* y *ABCC2* cuya variación genética ha resultados asociados con la eficacia en

pacientes con cáncer de mama tratados con docetaxel y doxorubicina respectivamente. Estos resultados nos sugieren que la variabilidad genética en estos transportadores de la familia ABC parece jugar un papel muy relevante dentro de la respuesta terapéutica, pudiendo ser excelentes candidatos en el estudio de otro tipo de tumoures que son tratados con fármacos transportados por estas mismas proteínas

6. Hemos identificado un SNP (rs3215400) en el promotor del gen *CDD* asociado con un riesgo superior de padecer sindrome de mano-pié inducido por capecitabina en pacientes de cáncer de mama y colorectal. Por otro lado, hemos encontrado que este SNP influye en la expresión de dicho gen por lo cual podría causar una mayor conversión de capecitabina a su forma activada, 5-FU, resultando en una mayor toxicidad.

7. Para la implementación en la clínica practica de las variantes identificadas a lo largo de esta tesis doctoral se requiere de la validación en nuevas series independientes a través de la creación de grandes consorcios donde se intégre la investigación con la clínica y así permitir la incorporación de estos predictores en los pacientes para la mejora del tratamiento.

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APPENDIX I

Publications derived from the thesis

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ORIGINAL ARTICLE

Common variations in *ERCC2* are associated with response to cisplatin chemotherapy and clinical outcome in osteosarcoma patients

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Platinum agents cause DNA cross-linking. Nucleotide excision repair genes play a key role in DNA damage repair. This study aims to investigate whether polymorphisms in these genes are associated with tumor response and survival in cisplatin-treated osteosarcoma patients. Eight single nucleotide polymorphisms in *ERCC2, XPC, XPA, ERCC1, ERCC4* and *ERCC5* genes were analyzed in 91 patients diagnosed with osteosarcoma and treated with cisplatin. A significant association with tumor response, after correction for multiple testing, was found for the Lys751Gln polymorphism in the *ERCC2* gene. We found that only 45% of patients with at least one polymorphic G allele responded compared with 80% of patients homozygous for the common T allele (odds ratio = 4.9, 95% confidence interval = 1.64–14.54, adjusted *P*-value = 0.047). In addition, carrying at least one ERCC2 Lys751GlnG allele was significantly associated with shorter event-free survival (median = 184 months, compared with 240 months for TT homozygotes; hazard ratio = 5.76, 95% confidence interval = 1.30–25.55; *P*-value = 0.021). Although ototoxicity was only recorded in 32 patients, we found weak evidence of an association with the CC genotype of XPC Lys939Gln (*P*-value = 0.042). This is the first pharmacogenetic study focused on osteosarcoma treatment providing evidence that polymorphic variants in DNA repair genes could be useful predictors of response to cisplatin chemotherapy in osteosarcoma patients.
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Keywords: cisplatin; single nucleotide polymorphisms; tumor response; osteosarcoma

Introduction

Osteosarcoma is one of the most frequent bone tumors, occurring mainly in young patients.[1] Standard treatment for osteosarcoma involves neoadjuvant therapy before surgical resection of the primary tumor, followed by post-operative chemotherapy.[2]

One of the most consistent prognostic factors for osteosarcoma is the histological response to pre-operative treatment,[3] evaluated as the percentage of tumor necrosis; which correlates with event-free survival (EFS) and overall survival.[4]

Despite chemotherapy and surgery, some (about 30%) patients relapse or metastasize.[5] As strategies such as the intensification of chemotherapy and the addition of other agents have not led to long-term benefits, many efforts are

directed at identifying factors that could predict drug response and clinical outcome.

Cisplatin is one of the chemotherapeutic agents most used in osteosarcoma therapy, together with doxorubicin and methotrexate.[2] Cisplatin causes DNA lesions by forming intra-strand and inter-strand cross-links that result in DNA distortion and inhibition of DNA replication.[6] The nucleotide excision repair (NER) pathway is one of the major DNA repair systems involved in the removal of platinum adducts.[7] The NER pathway is formed by a complex network of many proteins involved in lesion recognition, excision, DNA resynthesis and ligation.

Alterations in NER genes expression[8] as well as the presence of single nucleotide polymorphisms (SNPs)[9] are correlated with cisplatin resistance. SNPs in the *ERCC1* and *ERCC2*[10] genes have been found to be associated with platinum response in different clinical studies. In particular, the ERCC2 Lys751Gln variant was associated with reduced survival and worse prognosis in platinum-treated non-small-cell lung cancer[11] and colorectal cancer patients,[12] respectively.

In this study we investigated the relationship of eight SNPs in six NER genes to cisplatin response and survival in osteosarcoma patients.

Associations between these SNPs and cisplatin-induced hearing impairment (ototoxicity) were also explored.

Results

Study population

The clinical features of the 91 osteosarcoma patients are summarized in Table 1. The median age at diagnosis was 15 years (range 4 to 34 years). Only 15 patients were older than 18 years at the time of recruitment and 51 (56%) were male. Most (59%) of the osteosarcoma were osteoblastic, whereas 17% were chondroblastic. The remainders (24%) were grouped together as 'other'. At the time of diagnosis, 16% of the patients already presented metastasis, whereas 21% developed metastasis during follow-up. At the time of the final analysis on March 2007, the median follow-up was 91 months (range 10–272).

Single nucleotide polymorphism frequencies

The genotypic frequencies of the eight SNPs are shown in Table 2. All of them are common SNPs with minor allele frequencies between 0.19 and 0.46. There was no evidence of departure from the Hardy–Weinberg equilibrium for any of them.

Tumor response

Forty-two (60%) of the 70 patients for whom necrosis data were available were classified as good responders. The results of tumor response to treatment by genotype are shown in Table 2. A significant association was detected for the presence of at least one polymorphic allele of each of Lys751Gln in *ERCC2* and Lys939Gln in *XPC*. In particular, the polymorphic G allele of Lys751Gln was associated with a poor response: the estimated odds ratio (OR) under a dominant model was 4.89 (95% confidence interval (CI) = 1.64–14.54) (*P*-value = 0.004). We found that only 45% (18 of 40) of patients with at least one G allele were good responders compared with 80% (24 of 30) of patients homozygous for the T allele. On the contrary, the polymorphic C allele of XPC Lys939Gln was significantly associated with good response (OR = 0.34, 95% CI = 0.12–0.91, *P*-value = 0.032). For this SNP, 71% (29 of 41) of carriers of at least C allele responded to therapy compared with 45% (13 of 29) of patients homozygous for the A allele. No substantial changes were observed in these ORs after adjustment for each of cisplatin doses, tumor location and metastasis at diagnosis as covariates. Only the association with ERCC2 Lys751Gln polymorphism was maintained after correction for multiple testing (adjusted *P*-value = 0.047). No evidence of association was found for the other polymorphisms in the NER genes considered.

Table 1 Clinical characteristics of osteosarcoma patients (*N* = 91)

	Patients	
	No.	%
Age at diagnosis (years)		
Median		14.9
Range		3.7–34
Sex		
Female	40	44.0
Male	51	56.0
Subtype		
Osteoblastic	54	59.3
Chondroblastic	15	16.5
Other	22	24.2
Location		
Femur	46	50.5
Tibia	32	35.2
Arm	7	7.7
Central	6	6.6
Necrosis[a]		
Good	42	60.0
Poor	28	40.0
Metastasis		
No	57	62.6
At diagnosis	15	16.5
At follow-up	19	20.9
Status		
Alive	69	75.8
Dead	22	24.2
Relapse		
No	76	83.5
Yes	15	16.5

[a]Based on 70 osteosarcoma patients (see the methods section).

Table 2 Genotype distribution, logistic regression and Cox regression analyses assessing associations of polymorphisms with tumor response and event-free survival

Genotypes		Patients No (%)	Poor tumor response			Event-free survival		
			Odds ratio	95% CIs	P-value	Hazard ratio	95% CIs	P-value
ERCC2 Lys751Gln	*rs13181*							
TT		39 (42.9)	Referent			Referent		
TG		40 (44.0)	5.54	1.76–17.39	0.003	5.06	1.09–23.46	0.038
GG		12 (13.2)	3.20	0.65–15.70	0.152	8.33	1.52–45.56	0.014
TG/GG			4.89	1.64–14.54	0.004	5.76	1.30–25.55	0.021
ERCC2 Asp312Asn	*rs1799793*							
GG		39 (42.9)	Referent			Referent		
AG		42 (46.2)	1.52	0.54–4.28	0.431	1.71	0.50–5.87	0.390
AA		10 (11.0)	3.50	0.69–17.64	0.129	3.83	0.95–15.47	0.059
AG/AA			1.80	0.67–4.80	0.241	2.14	0.68–6.74	0.194
XPC Lys939Gln	*rs2228001*							
AA		36 (39.6)	Referent			Referent		
AC		43 (47.3)	0.28	0.10–0.84	0.023	1.05	0.33–3.33	0.931
CC		12 (13.2)	0.54	0.13–2.34	0.411	1.12	0.26–4.80	0.875
AC/CC			0.34	0.12–0.91	0.032	1.07	0.36–3.16	0.900
ERCC1 Lys504Gln	*rs3212986*							
GG		50 (54.9)	Referent			Referent		
GT		30 (33.0)	1.64	0.56–4.78	0.362	2.43	0.76–7.77	0.134
TT		11 (12.1)	2.51	0.63–10.05	0.194	2.05	0.49–8.65	0.328
GT/TT			1.87	0.71–4.94	0.206	2.30	0.78–6.75	0.129
ERCC1 Asn118Asn	*rs11615*							
TT		29 (31.9)	Referent			Referent		
CT		42 (46.2)	1.37	0.43–4.32	0.593	2.80	0.59–13.20	0.193
CC		20 (22.0)	1.78	0.48–6.62	0.391	3.35	0.64–17.48	0.151
CT/CC			1.50	0.51–4.37	0.457	2.98	0.67–13.27	0.151
ERCC4 Intron 1	*rs744154*							
CC		41 (45.1)	Referent			Referent		
CG		44 (48.4)	1.23	0.45–3.38	0.692	0.51	0.18–1.45	0.886
GG		6 (6.6)	2.70	0.38–18.96	0.318	0.00	0.00	0.977
GG			2.40	0.37–15.38	0.356	0.04	0.00–297.11	0.978
ERCC5 His46His	*rs1047768*							
CC		23 (25.3)	Referent			Referent		
TC		53 (58.2)	0.44	0.14–1.37	0.157	0.57	0.18–1.76	0.327
TT		15 (16.5)	0.34	0.07–1.77	0.201	0.55	0.11–2.84	0.474
TC/TT			0.42	0.14–1.26	0.123	0.57	0.19–1.67	0.301
XPA 5'UTR	*rs1800975*							
GG		45 (49.5)	Referent			Referent		
AG		38 (41.8)	0.75	0.27–2.09	0.577	1.90	0.62–5.83	0.261
AA		8 (8.8)	0.76	0.16–3.70	0.734	2.27	0.43–12.00	0.333
AG/AA			0.75	0.29–1.96	0.556	1.96	0.67–5.78	0.220

Abbreviation: CI, confidence interval.

Event-free survival

The polymorphic G allele of Lys751Gln was significantly associated with shorter EFS (hazard ratio under a dominant model = 5.76, 95% CI = 1.30–25.55, *P*-value = 0.021) (Table 2 and Figure 1). The median EFS of patients that carried the GG genotype was 141 months compared with 240 months for TT homozygotes.

No substantial changes were observed in this hazard ratio reported above after adjustment for each of cisplatin doses, tumor location, metastasis at diagnosis and tumor necrosis as covariates.

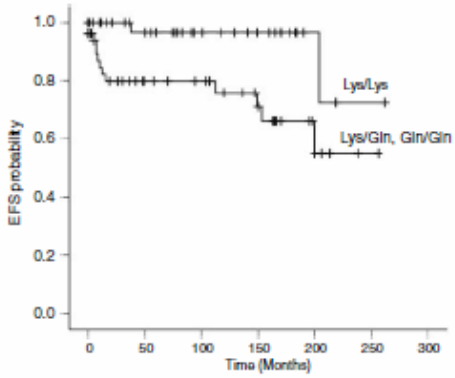

Figure 1 Kaplan-Meier curves for event-free survival (EFS) of osteosarcoma patients treated with platinum-based therapy. Analysis for ERCC2 Lys751Gln ($X^2 = 6.82$, P-value = 0.009).

Table 3 Logistic regression analysis for association of XPC Lys939Gln polymorphism with ototoxicity

% of patients with ototoxicity		Logistic regression		
		Odds ratio	95% CIs	P-value
XPC Lys939Gln				
AA	27% (3 of 11)	Referent		
AC	50% (8 of 16)	3.62	0.56–23.60	0.179
CC	80% (4 of 5)	17.16	1.10–266.78	0.042

Abbreviation: CI, confidence interval.

Association with ototoxicity

Data on ototoxicity were available for 32 patients. We detected a marginally significant association between this specific type of toxicity and rs2228001 (XPC) (Table 3). Ototoxicity was observed in 27% in patients with the AA genotype compared with 80% in patients with the CC genotype (OR = 17.16, 95% CI = 1.10–266.8, P-value = 0.042). The frequencies of the AA, AC and CC genotypes in this SNP were 20, 53 and 27%, respectively in 15 patients with ototoxicity, and 47, 47 and 6%, respectively in the 17 patients without hearing impairment. Sixty-seven percent of the patients with ototoxicity were good responders.

Discussion

The identification of molecular markers capable of predicting response to treatment is essential for osteosarcoma, as there are few alternative treatments for patients who relapse.[13] The only prognostic factors available are the presence of metastasis, tumor necrosis after neoadjuvant chemotherapy, tumor location and tumor volume.[2] Many studies are now focused on the identification of molecular prognostic markers, and molecular tumor profiling in particular, in order to improve the treatment of these tumors. To date, some pathways, such as CXCR4, survivin, MMP2 and MMP9, have been related to osteosarcoma patient outcome.[14] Nevertheless, the genetic profile of the patients may also play a role in treatment response, so it would be of great interest also to explore the genetic variations in patients with osteosarcoma. Cisplatin is one of the most effective chemotherapeutic agents used for osteosarcoma treatment. As the NER pathway is responsible for the removal of DNA adducts induced by platinum compounds,[6] we analyzed the association between treatment response and polymorphisms in the NER genes *ERCC2*, *XPC*, *ERCC1*, *ERCC4*, *ERCC5* and *XPA* in osteosarcoma patients treated with cisplatin.

In our study, carrying at least one G allele in ERCC2 Lys751Gln conferred an estimated fivefold risk of poor tumor response in osteosarcoma patients treated with cisplatin. This result was consistent with that observed for survival, with nearly fivefold increased risk of relapse or death.

The association between the ERCC2 Lys751Gln polymorphism and platinum response has already been described.[10,12,15]

On the contrary, some reports in lung cancer didn't find an association between this polymorphism and cisplatin response;[16–18] further studies to elucidate the real role of ERCC2 Lys751Gln polymorphism on cisplatin response are warranted. Regarding the other variant in the *ERCC2* gene, the ERCC2 Asp312Asn polymorphism, we observed a trend of an increased risk of poor tumor response and reduced EFS, but without reaching the statistical significance.

Ruzzo *et al.*[12] also studied ERCC2 Asp312Asn, and found that the association with platinum response seemed to be weaker than for Lys751Gln, located 12 kb downstream. In our patient series, we have observed a similar trend of a weaker effect for Asp312Asn polymorphism. This finding could be explained by the fact that these two SNPs are in incomplete linkage disequilibrium ($D' = 0.66$) in our sample. Therefore, our result suggests that the ERCC2 Lys751Gln polymorphism could be a better predictive marker for both clinical response and survival.

The mechanisms by which this polymorphism affects platinum response are not fully clear. The *ERCC2* gene encodes a DNA helicase that is an essential component of the NER pathway. The effect of this polymorphism on DNA repair has been evaluated by functional studies with controversial results. Lunn *et al.*[19] showed that carriers of the common T allele had a suboptimal DNA repair activity, whereas Spitz *et al.*[20] reported that the polymorphic G variant was associated with lower DNA repair activity. On the other hand, some reports didn't find any evidence of association between this polymorphism and DNA repair activity.[21,22] The discrepancies observed between these studies could be because of the different assays used to measure DNA repair. As they measure different parameters,

such as X-ray-induced chromatic aberrations and the host cell reactivation assay, they may not be appropriate for the evaluation of the effect of this polymorphism on DNA repair. Another possible explanation is that this polymorphism is not causal itself, but rather is in linkage disequilibrium with a functional polymorphism involved in cisplatin response. ERCC2 Lys751Gln is a coding SNP located in the last exon, close to the 3′UTR region. Variation in this region could affect the stability of mRNA or even the regulation of protein translation.

Although after conservative multiple testing correction the significant association is lost, our results suggest a possible role of XPC in platinum response. This gene encodes a protein of 940 amino acids that recognizes DNA damage, and XPC polymorphisms have earlier been related to cancer risk.[23,24] Additionally, the minor allele of XPC Lys939Gln could be associated with higher risk of ototoxicity, although the limited number of patients included in the analysis does not permit definitive conclusions. Although a recent paper[25] has reported an association of polymorphisms in the drug metabolism gene, *GSTP1*, with ototoxicity in testicular cancer survivors,[25] no studies relating XPC variation with hearing impairment have been published to date. A possible explanation for our observed association could be that Lys939Gln may reduce the activity of XPC, and thus its DNA repair capacity. This decrease in DNA repair capacity could enhance apoptosis in response to platinum in both tumor cells, increasing the cisplatin response, as well as in the normal outer hair cells of the organ of Corti, explaining the associated ototoxicity. Unfortunately, functional studies for this polymorphism have also yielded inconsistent results.[26,27] Additional studies are required to evaluate the role of XPC variations in cisplatin response and ototoxicity.

Despite the earlier published data reporting an association between ERCC1 SNPs and platinum response,[28,29] there was no strong evidence of this effect in our patients. We only observed a tendency towards an increased risk of poor tumor response and reduced EFS in patients carriers of the polymorphic alleles for ERCC1 Lys504Gln and ERCC1 Asn118Asn polymorphisms, but without reaching the statistical significance in none of the cases. The rest of variants analyzed in *XPA*, *ERCC5* and *ERCC4* genes were included in this study since previously described as cancer risk related variants[24,30,31] that could be an indication of the implication of these variants in DNA repair efficiency. Despite so, we have not found any evidence of association with cisplatin response.

We have to highlight that because of the multidrug neoadyuvant therapy, the response and the outcome of the patients may be the result of the combination of all these drugs, and we cannot exclude the fact that the polymorphisms could be relevant to the response to some of the other agents.

However, to date, there is no clear evidence in the literature of an association between NER and sensitivity to the other drugs used in combination with cisplatin in osteosarcoma. In fact, the NER pathway removes cisplatin-induced DNA adducts[7] and, to date, doesn't seem to be related to the damage induced by these other drugs. Therefore, the association found in our study with NER gene polymorphisms could be attributed to cisplatin drug administration in particular.

In conclusion, to our knowledge, this is the first study showing the involvement of polymorphisms in DNA repair genes in the response of osteosarcoma patients to chemotherapy. We found that polymorphisms in the *ERCC2* gene, specifically ERCC2 Lys751Gln, could be a marker for predicting the tumor response and clinical outcome of osteosarcoma patients. Furthermore, for the first time we have found a possible evidence of a role of XPC in platinum response. However, further studies with larger number of patients are required to confirm our findings. Functional analyses for these associated SNPs are also required to elucidate their role in DNA repair activity.

Patients and methods

Patients, treatments and clinical variables

This retrospective study included 91 patients diagnosed with osteosarcoma and treated at the University Clinic of Navarra, Pamplona, Spain between 1986 and 2007. The study was approved by the ethics committee of the University Clinic.

Patients were treated preoperatively with intravenous (i.v.) adriamycin (three courses at 25–30 mg m^{-2} per day for 3 days), i.v. methotrexate (four courses of up to 14 g m^{-2} per day for 1 day) and intra-arterial cisplatin (three courses at 35 mg m^{-2} per day for 3 days) and, after surgery, the adjuvant chemotherapy included methotrexate (10 g m^{-2} per day for 1 day) and alternate cycles of i.v. cisplatin/adriamycin or i.v. actinomycin D (0.45 mg m^{-2} per day for 3 days), cyclophosfamide (500 mg m^{-2} per day for 3 days) and vincristine (1.5 mg m^{-2} per day for 1 day) up to 48 weeks of treatment.

The cumulative dose of platinum relative to the total body surface was recorded for each patient, considering both intra-arterial neoadjuvant and i.v. adjuvant treatment with cisplatin. Cisplatin was given at a dose ranging from 120 to 1131 mg m^{-2} for intra-arterial treatment and from 83 to 948 mg m^{-2} for i.v. treatment.

Treatment response was determined histologically by the percentage of necrosis induced in the tumor after neoadjuvant chemotherapy. Patients with <90% necrosis were classified as poor responders and those with 90% necrosis or higher, as good responders.[32]

Of the 91 osteosarcoma patients, 16 were excluded because the neoadyuvant regimen did not include cisplatin, and a further five lacked tumor response information. Therefore, a total of 70 were considered in the analysis of response to treatment.

Event-free survival was considered from tumor diagnosis to the first of disease recurrence, development of lung or bone metastases and/or death. Patients who were alive and free of disease at the last follow-up evaluation (March 2007)

were censored at that time. Data for EFS were available for all the 91 patients included in the study.

Ototoxicity was evaluated using objective audiometric tests at the otorhynolaryngology consultation.

Other clinical data, including age, sex, tumor location, metastatic events (both at diagnostic or at follow-up) and relapses (disease recurrence in the same bone) were systematically recorded from the clinical records. Only conventional high-grade osteosarcomas were included, regardless of metastatic stage at diagnosis.

DNA extraction and genotyping

The peripheral blood samples were collected from patients in remission, with their informed consent, and data were encrypted, anonymized and linked to their clinical data. All blood samples were chemonaïve, as they were obtained either at the time of first consultation before chemotherapy or at long-term remission. Only in one case the sample was collected after the first cycle of chemotherapy.

Genomic DNA was extracted from the peripheral blood lymphocytes, using standard protocols of phenol–chloroform extraction. DNA was quantified using PicoGreen (Invitrogen Corp., Carlsbad, CA, USA) and diluted to a final concentration of 5 ng μl^{-1} for genotyping. A total of 10 ng of DNA were used for each genotyping reaction.

Eight SNPs located in six DNA repair genes were analyzed: Lys751Gln (rs13181) and Asp312Asn (rs1799793) in *ERCC2* (excision repair group 2), Lys939Gln (rs2228001) in *XPC* (xeroderma pigmentosum group C), Lys504Gln (rs3212986) and Asn118Asn (rs11615) in *ERCC1* (excision repair group 1), rs744154 in *ERCC4* (excision repair group 4) intron 1, His46His (rs1047768) in *ERCC5* (excision repair group 5) and rs1800975 in *XPA* 5′UTR (xeroderma pigmentosum group A) genes. All these SNPs have been related to platinum and radiotherapy response and/or risk of cancer in other type of tumors.[9–12,24,30,31] Genotypes were determined by TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) for all SNPs, except rs1800975 (or which the KASPar SNP genotyping system was used (KBioscience, Hoddesdon, UK). Allelic discrimination was carried out using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Duplicate samples were genotyped as a quality control.

Statistical analysis

Associations between SNPs and platinum response were assessed using logistic regression analysis,[33] comparing genotype frequencies in good responders and poor responders, and estimating odds ratios. Homozygotes for the most frequent allele were used as the reference group. In addition to the model comparing the genotypes separately (co-dominant model), we considered dominant and recessive models, the best fitting model determined by parsimony. *P*-values were corrected for multiple comparisons using a permutation test. We randomly assigned the status of responder/non-responder, and recorded the minimum *P*-value from co-dominant, dominant and recessive models among all SNPs considered. This was repeated 10 000 times to generate the empirical distribution of minimum values, and the proportion less than a given observed *P*-value was considered the corresponding adjusted *P*-value.

Single nucleotide polymorphisms showing evidence of an association with tumor response were then assessed in relation to event-free survival using Cox regression analysis.[34]

Tumor location (femur, tibia, arm and central), cisplatin cumulative dose (continuous) and metastasis at diagnosis were included as covariates in multivariable logistic regression and Cox regression analyses.

The SPSS software (version.13.0, SPSS Inc., Chicago, IL, USA) was used for all analyses, and *P*-values <0.05 were considered statistically significant.

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Conflict of interest

The authors declare no conflict of interest.

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Clinical Cancer Research

Predictive Biomarkers and Personalized Medicine

A Polymorphism in the *Cytidine Deaminase* Promoter Predicts Severe Capecitabine-Induced Hand-Foot Syndrome

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Abstract

Purpose: Hand-foot syndrome (HFS) is one of the most relevant dose-limiting adverse effects of capecitabine, an oral prodrug of 5-fluorouracil used in the standard treatment of breast and colorectal cancer. We investigated the association between grade 3 HFS and genetic variations in genes involved in capecitabine metabolism.

Experimental Design: We genotyped a total of 13 polymorphisms in the carboxylesterase 2 (*CES2*) gene, the cytidine deaminase (*CDD*) gene, the thymidine phosphorylase (*TP*) gene, the thymidylate synthase (*TS*) gene, and the dihydropyrimidine dehydrogenase (*DPD*) gene in 130 patients treated with capecitabine. We correlated these polymorphisms with susceptibility to HFS.

Results: We found an association of HFS appearance with rs532545 located in the promoter region of *CDD* (OR = 2.02, 95% CI = 1.02–3.99, $P = 0.039$). Because we found no association between the *rs532545* genotype and CDD mRNA expression in Epstein-Barr virus lymphoblastoid cells, we explored additional genetic variations across the *CDD* promoter. We found an insertion, rs3215400, in linkage disequilibrium with rs532545 ($D' = 0.92$), which was more clearly associated with HFS (OR = 0.51, 95% CI = 0.27–0.95, $P = 0.028$) in patients and with total *CDD* gene expression ($P = 0.004$) in lymphoblastoid cells. *In silico* analysis suggested that this insertion might create a binding site for the transcriptional regulator E2F. Using a SNaPshot assay in lymphoblastoid cells, we observed a 5.7-fold increased allele-specific mRNA expression from the deleted allele.

Conclusions: The deleted allele of rs3215400 shows an increased allele-specific expression and is significantly associated with an increased risk of capecitabine-induced HFS. *Clin Cancer Res; 17(7); 2006–13. ©2011 AACR.*

Introduction

Capecitabine (Xeloda) is a 5-fluorouracil (5-FU) prodrug widely used in the treatment of breast and colorectal cancer (1). The conversion takes place through a 3-step enzymatic process: after activation by carboxylesterase 2 (CES2) and cytidine deaminase (CDD), capecitabine is converted to 5-FU by thymidine phosphorylase (TP), which is highly expressed in tumors and liver. One of the main targets of 5-FU is TS (thymidylate synthase). Finally, 5-FU is catabolized by DPD (dihydropyrimidine dehydrogenase; ref. 2).

Capecitabine offers a more selective alternative to 5-FU, as it is converted into the active form specifically in the tumor cell (2), lowering the adverse effects related to 5-FU (3). Even so, hand-foot syndrome (HFS) occurs in a large percentage (almost 30%) of capecitabine-treated patients. HFS is a side effect of continuous 5-FU infusion and of other drugs such as doxorubicin, cytarabine, and docetaxel (4). It is characterized by tenderness, redness, and swelling of palms and soles and often necessitates dose reduction or even treatment interruption (5).

Pharmacogenetics aims to understand the relationship between genetic variation and adverse drug reaction (ADR) or treatment response. Genetic analysis of genes involved in the metabolism of anticancer drugs is becoming more and more important for elucidating the interpatient pharmacodynamic variability of anticancer drugs.

With regard to capecitabine, some polymorphisms such as three 28-bp repeats in the 5′ untranslated region (UTR) of the *TS* gene containing a G>C mutation in the second repeat, a 6-bp deletion in the 3′ region of the *TS* gene, and the inactivating mutation *IVS14+1G>A* in the *DPD* gene

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Translational Relevance

This article shows evidences of a role of a *CDD* genetic variant in capecitabine-induced severe hand-foot syndrome appearance, suggesting that this variant might be useful to prevent this adverse event in patients treated with this drug. These findings give new insights to elucidate the mechanisms of capecitabine-related toxicity and could help in individualizing the treatment.

(6–8) have been related to severe global toxicity appearance when ADRs such as myelosuppression, hematologic toxicity, diarrhea, and HFS are grouped.

The aim of the present study was to investigate the relationship between capecitabine-induced HFS and polymorphisms in genes of the capecitabine metabolic pathway (*CES2*, *CDD*, *TP*, and *DPD*) and target gene (*TS*).

Methods

Study subjects

This retrospective study included 130 patients with a diagnosis of breast cancer or colorectal cancer treated at the Hospital Universitario San Carlos, Madrid, Spain, between June 2005 and March 2009. The study was conducted according to the Declaration of Helsinki and approved by the Ethics Committee of the institution. All patients signed an informed consent. Most (72%) of the patients were diagnosed with breast cancer, whereas the remaining patients had colorectal cancer. The median age at diagnosis was 63 years (range = 28–88 years), and 112 patients (86%) were female.

Capecitabine was administered according to 2 different schedules. Colorectal cancer patients were treated with a standard regimen (1,250 mg/m^2 orally every 12 hours on days 1–14 every 3 weeks), whereas breast cancer patients were treated either with the same standard regimen or with a continuous regimen (800 mg/m^2 orally every 12 hours daily).

HFS was graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (version 2). Grade 3 HFS was defined as skin changes with pain interfering with function. For study purposes, we used as study endpoint the maximum HFS grade experienced by the patients along the treatment, considering grade 0–2 of HFS as no or low toxicity and grade 3 as high toxicity. Other clinical data were recorded such as age, sex, capecitabine regimen, number of reductions, and hepatic metastasis (as a variable that could interfere with capecitabine metabolism; Table 1).

Table 1. Clinical characteristics of patients ($N = 130$)

	Patients	
	n	%
Age at diagnosis, y		
Median	63	
Range	28–88	
Sex		
Female	112	86
Male	18	14
Diagnostic		
Breast cancer	93	72
Colorectal cancer	37	28
Stage		
I	3	2
II	9	7
III	33	25
IV	85	65
Treatment setting		
Postsurgical adjuvant	39	30
First-line metastatic	26	20
Second-line metastatic	13	10
Third-line metastatic or further	52	40
Capecitabine		
Standard	104	80
Continuous	26	20
No. of capecitabine reductions		
0	59	45
1	54	42
≥2	17	13
HFS		
Grade 0	41	31
Grade 1	23	18
Grade 2	25	19
Grade 3	41	32
Hepatic metastasis		
No	78	60
Yes	51	40

Genotyping

A total of 13 polymorphisms located in 5 different genes were analyzed in the 130 breast and colorectal cancer patients. Eleven polymorphisms were located in capecitabine metabolic pathway genes. In the *CDD* gene, single nucleotide polymorphisms (SNP) rs532545 −451C > T, rs602950 −92A > G, both located in the promoter, and Lys27Gln, the coding SNP rs2072671 (9–13); in the *DPD* gene, the intronic SNP rs3918290, IVS14+1G>A, in the splice donor site flanking exon 14 that causes exon skipping and inactivation of DPD allele (7, 14–16); in the *CES2* gene, rs2241409, rs11568314, rs11568311, all intronic SNPs, and rs11075646 823C > G; in the *CES2* gene promoter and in the *TP* gene, the intronic SNP rs470119 and the coding SNPs rs11479 Ser471Leu and rs131804 Ala324Ala. The remaining 2 polymorphisms were located in the 5-FU target gene *TS*, a 28-bp double- or triple-tandem repeat, including a G > C mutation in the 5′ region and a 6-bp deletion in the 3′ region (6, 14, 17).

All polymorphisms have been described in the literature as possible functional variants except in the TP and CES2 genes, for which, because of the lack of candidate functional SNPs, tagSNPs were selected.

Genomic DNA was extracted from peripheral blood lymphocytes by automatic DNA extraction (MagNA Pure; Roche) according to the manufacturer's protocol. DNA was quantified using PicoGreen (Invitrogen Corp.).

Genotypes were determined using the KASPar SNP genotyping system (KBioscience). Allelic discrimination was carried out using the ABI PRISM 7900 Sequence Detection System (Applied Biosystems).

For the polymorphisms in the TS gene, restriction fragment length polymorphisms (RFLP) and direct sequencing were used. In particular for the 5′-UTR polymorphism, a fragment containing the 28-bp repeat was amplified starting from 100 ng genomic DNA, using the following primers: 5′-TTCCCGGGTTTCCTAAGACT-3′ and 5′-TGGATCTGCCCCAGGTACT-3′. PCR products were purified by ExoSap-IT (USB Corporation) and directly sequenced using an ABI3730 DNA analyzer (Applied Biosystems). 3′-UTR variation was analyzed by RFLP as described (6).

Duplicate and negative samples were included in all assays.

Fine mapping at the *CDD* promotor

A 959-bp fragment of the promoter was amplified from 30 healthy controls, using the following primers: 5′-ATGCAGTGGTGCAATCTGAG-3′ and 5′-GTGCCCACCTTTACCTTTGA-3′. After purification, DNA fragments were sequenced as described earlier.

Lymphoblastoid cell cultures

Eighty-nine lymphoblastoid cell lines derived from the Caucasian Utah CEPH lines were purchased from the Coriell Institute for Medical Research (Camden, NJ). Lymphoblastoid cell lines were cultured in RPMI 1640 containing 15% FBS (Euroclone) and maintained in a humidified incubator at 37°C and 5% CO_2.

Genomic DNA and total RNA were extracted from exponentially growing cells, using DNAZol (MBC; Molecular Research Center, Inc.) and TRIzol (Invitrogen), respectively, according to the manufacturer's protocol. DNA was genotyped for rs532545, rs602950, rs2072671, rs3215400, and rs603412 SNPs by Kaspar Genotyping Assays as described earlier.

Gene expression assay

One microgram of RNA was reverse transcribed using an oligo(dT)$_{18}$ primer and Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's recommendations. Real-time PCR was carried out in the 89 cell lines by using the TaqMan Gene Expression Assays (Applied Biosystems), following the manufacturer's protocol (Hs00156401_m1 probe for CDD) on an ABI PRISM 7900 Sequence Detection system (Applied Biosystems). The glyceraldehyde 3-phosphate dehydrogenase transcript level was used as a reference (Hs9999905_m1). Data were analyzed using absolute quantification on resulting C_t (cycle threshold) values generated on the sequence detection system. Serial dilutions of cDNA of a control sample were used to generate standard curves, and quantity mean (Qty mean) for each sample was calculated. Each sample was evaluated in triplicate.

Prediction of transcription factor binding sites

We carried out *in silico* prediction of putative transcription factor binding sites by using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html, v1.3) and Jaspar (http://jaspar.cgb.ki.se/).

Allele-specific expression assay

Allele-specific mRNA expression was measured amplifying a 244-bp region around SNP rs3215400 in the 43 Caucasian lymphoblastoid cell lines heterozygous for this SNP. The following primers were used: 5′-AAAGCTGCGTACCTGAGAGC-3′ and 5′-TGACTGTAGGGGCAGTAGGC-3′.

cDNA was prepared from RNA treated with DNase I (Ambion), using Superscript II Reverse Transcriptase (Invitrogen) with random hexamers according to the manufacturer's recommendations.

Primer extension with fluorescent dideoxynucleotides was done using the SNaPshot System (Applied Biosystems), followed by capillary electrophoresis on an ABI3730 DNA analyzer (Applied Biosystems). Data were analyzed by Peak Scanner 1.0 software (Applied Biosystems).

Allele-specific expression (ASE) ratios were calculated as follows: the ratio between the 2 alleles' peak area of cDNA was divided by the same ratio for genomic DNA. Each sample was assayed using 2 independent cDNA preparations; 2 independent single base extensions were run for each cDNA preparation, giving a total of 4 replicates. The SNaPshot ASE variation value for each individual is given as the average of the 4 analyses.

Statistical analysis

The main objective of the study was to assess the correlation between SNP variations and severity of HFS (low or grades 0–2, vs. high or grade 3 + 4). Associations between SNPs and HFS were assessed using logistic regression analysis, comparing genotype frequencies in patients with low toxicity and high toxicity, and estimating ORs. Individuals who were homozygous for the most frequent allele were used as the reference group.

Sex, age (continuous), tumor type (colorectal and breast cancer), capecitabine dose (continuous), and number of dose reductions were included as covariates in multivariate logistic regression.

In addition to the model comparing the genotypes separately (codominant model), we considered log-additive, dominant, and recessive models.

Associations between gene expression and SNPs in cell lines were assessed by ANOVA when considering the 3

genotypes separately and Student's *t* test, assuming equal variances considering the wild-type homozygous versus the polymorphic heterozygous and homozygous genotypes. All expression data (Qty mean) were $\log_2$ transformed to obtain normally distributed data.

The R (version 2.6.0.2) and the SPSS software (version 13.0, SPSS Inc.) were used for all analyses, and the values of $P \geq 0.05$ were considered statistically significant. We estimated that at a nominal statistical significance level of 0.05, our study had 80% power to detect ORs under a dominant model of at least 3.0 for all but the less frequent SNPs (rs11568314, rs11568311, and rs11479) for which the minimum OR detectable was 6.0.

Results

The polymorphisms analyzed and the genotypic frequencies are shown in Table 2. The minor allele frequencies (MAF) were between 0.06 and 0.42. There was no evidence of departure from the Hardy–Weinberg equilibrium for any of them. Call rate was more than 99% for all samples, and duplicated samples showed an extremely high concordance (>99%). Grade 3 HFS was observed in 41 (32%) of the 130 patients treated with capecitabine.

We found a significant association with HFS only for a polymorphism in the *CDD* gene. In particular, the polymorphic T allele of rs532545 was associated with higher incidence of grade 3 HFS: the estimated OR was 2.02 ($P = 0.039$). No substantial changes were observed in this OR after adjustment for capecitabine dose, tumor type, age, and hepatic metastasis, whereas adjustment for dose reduction increased the significance of the association. No evidence of association was found for the polymorphisms in the other genes considered (Table 2).

We found only 1 patient heterozygous for the *DPD* SNP rs3918290, and this patient experienced life-threatening toxicities (severe myelosupression and mucositis). This SNP was not included in the analyses. Lethal outcome or high toxicity has been reported after treatment with 5-FU or capecitabine (8, 16, 18).

Because of the SNP associated with HFS is located in the promoter of the *CDD* gene and had been described to affect transcription by Fitzgerald and colleagues (10), we analyzed the relationship with CDD mRNA levels by quantitative real-time PCR. Because RNAs were not available for our patients, 89 lymphoblastoid cell lines from Caucasian healthy individuals were used for this purpose. After genotyping of rs532545 (MAF = 0.29) in these cell lines, we found that this SNP was not associated with a significant change in mRNA levels ($P = 0.671$).

Neither of the other 2 SNPs in the *CDD* gene analyzed (rs602950, MAF = 0.29; rs2072671, MAF = 0.36) was associated with gene expression (*P* values of 0.655 and 0.327 for rs602950 and rs2072671, respectively).

We hypothesized that rs532545 might not be the causal SNP but simply a marker SNP, so we searched by fine mapping of the promoter for other variants that showed stronger association with HFS. We sequenced a 959-bp fragment at the 5′ extreme of the *CDD* gene and found, apart from the 3 SNPs originally included in the study (rs602950, rs532545, and rs2072671), 2 more common variants already annotated in the dbSNP database (rs3215400 and rs603412). We genotyped our series of 130 patients for these 2 variants and found a statistically significant association with HFS for rs3215400 (Table 3). In particular, carriers of at least one inserted C allele of rs3215400 had lower risk of developing grade 3 HFS (OR = 0.37, $P = 0.020$) compared with individuals homozygous for the deleted allele. Further analysis was carried out for this variant, comparing grade 0 versus 3 to better discriminate the HFS phenotype. Although the sample size was greatly reduced ($N = 96$), the statistical significance was maintained ($P = 0.045$). This variant is in linkage disequilibrium (LD) with the previously associated rs532545 ($D' = 0.92$), and the LD block structure under the association interval is given in Figure 1A. To see whether the effect we observed was due to a single variant or to a combination of the 2 associated polymorphisms, rs532545 and rs3215400, we also analyzed the effect of the haplotypes on HFS. We found that the haplotypes that conferred an increased risk of developing HFS were only those that contained the deleted allele of the rs3215400 variant (data not shown).

We genotyped by the same approach the 2 polymorphisms we found after promoter sequencing, rs3215400 (MAF = 0.44) and rs603412 (MAF = 0.44), in the 89 lymphoblastoid cell lines. Again, we investigated the correlation with gene expression and found that the rs3215400 variant was associated with a significant difference in CDD mRNA levels. The median expression in cell lines with the homozygous *del-del* genotype was 3.1-fold higher than that of those with *del-C* heterozygous and *CC* homozygous genotypes ($P = 0.004$; Fig. 1B). The other *CDD* variant rs603412 was not significantly associated with gene expression ($P = 0.4$).

To elucidate whether the associated rs3215400 marker could be a putative functional variant, we carried out an *in silico* analysis with two different computer tools for the prediction of transcription factor binding sites and we found that the deleted allele of rs3215400 abrogates a binding site for the transcription factor E2F (Fig. 2A). Because of the global gene expression association and location in a putative transcriptional element, a more comprehensive analysis was carried out. Specifically, we measured ASE by SNaPshot in both genomic DNA and cDNA of the 43 cell lines heterozygous for rs3215400. As expected, the allelic ratio for genomic DNA was around 1, ranging from 0.95 to 1.6; in contrast, all samples analyzed displayed an increased allele ratio in cDNA compared with genomic DNA (Fig. 2B). In all cell lines analyzed, the ASE ratio for the deleted allele versus the C allele was more than 3, with an average value of 5.7 (range = 3.9–7.7; SD = 0.83).

Table 2. Genotype distribution and logistic regression analyses assessing associations of polymorphisms with grade 3 HFS

Genotype	*n* (%)		OR	95% CI	*P*
	Grade 0–2 HFS	Grade 3 HFS			
CDD rs532545					
Dominant					
CC	37 (41.6)	11 (26.8)	Referent		
CT/TT	52 (58.4)	30 (73.2)	2.28	0.95–5.44	0.057
Additive			2.02	1.02–3.99	0.039
CDD rs602950					
Dominant					
AA	37 (41.6)	13 (31.7)	Referent		
AG/GG	52 (58.4)	28 (68.3)	1.82	0.79–4.22	0.153
Additive			1.75	0.90–3.40	0.094
CDD rs2072671					
Dominant					
AA	36 (40.4)	13 (31.7)	Referent		
AC/CC	53 (59.6)	28 (68.3)	1.72	0.74–3.97	0.201
Additive			1.55	0.80–2.99	0.190
CES2 rs2241409					
Dominant					
CC	52 (58.4)	26 (63.4)	Referent		
CT	37 (41.6)	15 (36.6)	0.71	0.31–1.60	0.403
Additive			0.78	0.40–1.52	0.456
CES2 rs11568314					
Codominant					
AA	78 (87.6)	37 (90.2)	Referent		
AT	11 (12.4)	4 (9.8)	0.74	0.20–2.71	0.648
CES2 rs11568311					
Codominant					
GG	76 (85.4)	36 (87.8)	Referent		
GA	13 (14.6)	5 (12.2)	0.81	0.25–2.60	0.715
CES2 rs11075646					
Dominant					
CC	69 (77.5)	29 (70.7)	Referent		
CG/GG	20 (22.5)	12 (29.3)	1.43	0.59–3.47	0.434
Additive			1.32	0.62–2.85	0.475
TP rs470119					
Codominant					
GG	38 (43.2)	19 (46.3)	Referent		
GA/AA	50 (56.8)	22 (53.7)	0.91	0.41–2.00	0.811
Additive			0.94	0.52–1.70	0.846
TP rs131804					
Codominant					
AA	32 (36.0)	14 (34.1)	Referent		
AG/GG	57 (64.0)	27 (65.9)	1.09	0.48–2.46	0.842
Additive			1.03	0.57–1.85	0.927
TP rs11479					
Codominant					
CC	75 (86.2)	37 (90.2)	Referent		
CT	12 (13.8)	4 (9.8)	0.76	0.21–2.68	0.664
TS 3′-UTR					
Dominant					
6bp/6bp	34 (38.2)	22 (53.7)	Referent		
6bp/del	55 (61.8)	19 (46.3)	0.55	0.25–1.21	0.138
Additive			0.67	0.37–1.21	0.172
TS 5′-UTR					
Dominant					
2R2R/2R3RC/3RC3RC	54 (62.1)	26 (63.4)	Referent		
2R3RG/3RC3RG/3RG3RG	33 (37.9)	15 (36.6)	1.10	0.49–2.49	0.821
Additive			1.02	0.53–1.93	0.960

Table 3. Genotype distribution and logistic regression analyses assessing associations of rs3215400 and rs603412 with grade 3 HFS

Genotype	*n* (%)		OR	95% CI	*P*
	Grade 0–2 HFS	Grade 3 HFS			
CDD rs3215400 943insC					
Dominant					
– –	23 (25.8)	19 (46.3)	Referent		
–C/CC	66 (74.2)	22 (53.7)	0.37	0.16–0.86	0.020
Additive			0.51	0.27–0.95	0.028
CDD rs603412 −205C>G					
Dominant					
CC	28 (31.8)	12 (29.3)	Referent		
CG/GG	60 (68.2)	29 (70.7)	1.19	0.50–2.80	0.693
Additive			1.41	0.77–2.60	0.261

Discussion

In the present study, we used a candidate pathway approach that analyzes polymorphisms across the genes responsible for metabolizing capecitabine and found an association between rs3215400 polymorphism in the *CDD* gene and grade 3 HFS.

The *CDD* gene encodes an enzyme involved in the pyrimidine salvage pathway and catalyzes irreversibly the hydrolytic deamination of cytidine and deoxycytidine to their corresponding uridine derivatives (10). In addition, *CDD* plays an essential role in the metabolism of a number of antitumor cytosine nucleoside analogues, leading to their pharmacologic activation to 5-FU.

To date, several studies have addressed the relationship between polymorphisms in the *CDD* gene and sensitivity to cytosine nucleoside analogues or related toxicities. The G208A polymorphism has been reported to be associated with 1β-D-arabinofuranosylcytosine sensitivity (19) and gemcitabine-related toxicities in the Japanese population (20), but it was not included in our study because it is almost monomorphic in the Caucasian population (12, 13). The 79A>C variant (rs2072671), which we did include in our study, has been reported to be related to gemcitabine sensitivity, but there is no clear evidence of a functional role or association with toxicities (19). Fitzgerald and colleagues (10) reported a difference in *CDD* expression considering haplotypes containing rs602950 and rs532545, but because this haplotype is very rare (≥1%) in our series of patients and in cell lines, it cannot account for the differences in expression observed. To our knowledge, only Ribelles and colleagues (9) have analyzed the association between the rs3215400 *CDD* polymorphism and HFS, but not significant correlation was found. Because of the limited sample size in the study of Ribelles and colleagues, their negative result could be a consequence of a lack of statistical power. Although the present study has been conducted using a limited number of patients as well, the further functional study conducted in cell lines

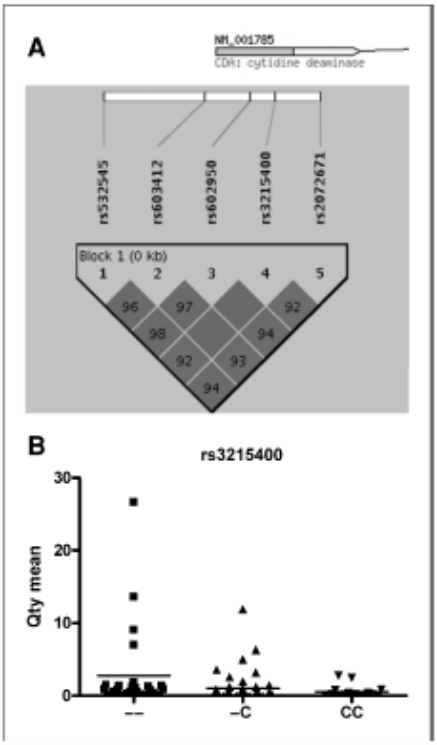

Figure 1. A, LD among the 5 variants studied at the *CDD* gene. Pairwise LD measures (D') calculated with the software package Haploview (version 4.1) are shown. B, effect of the rs3215400 *CDD* polymorphism on gene expression in Coriell lymphoblastoid cell lines.

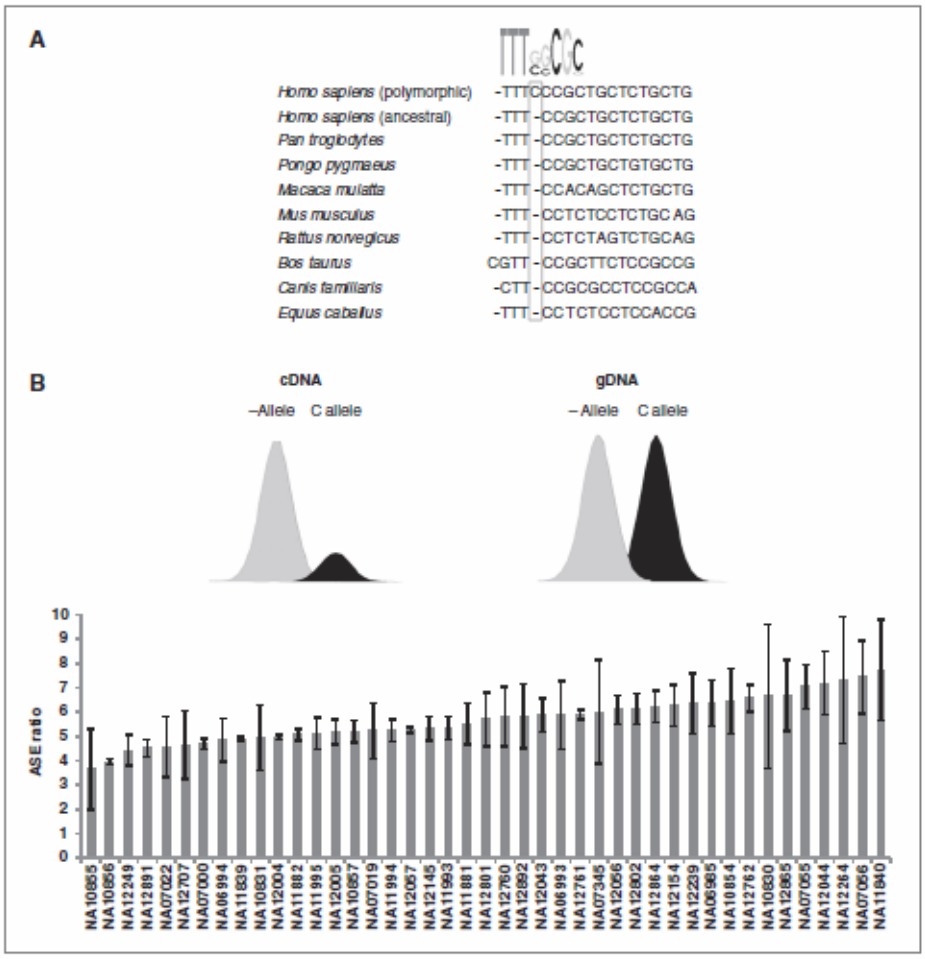

Figure 2. A, transcription factor binding site prediction: the rs3215400 *del* allele abrogates the E2F binding site. B, ASE analysis by SNaPshot in 43 Hapmap cell lines heterozygous for rs3215400. Top, an example of the peaks obtained for the deleted allele (gray) and for the C allele (black) for cDNA (left) and for genomic DNA (gDNA; right). The ASE (bottom) ratio was calculated by normalizing the ratio between the peak areas of the 2 alleles in cDNA for the same ratio in the genomic DNA.

supports our finding showing that the association observed in patients probably reflects the effect of this regulatory variant on *CDD* expression. Of the 5 common variants we analyzed at the *CDD* locus, rs3215400 found to be the most strongly associated with HFS. We hypothesized, based on *in silico* analysis, that the presence of a C allele at position 943 of the *CDD* gene is critical for transcriptional suppression of the *CDD* gene resulting in reduced CDD production and that such suppression is mediated through E2F binding. Such a functional explanation is in agreement with our observation that the deleted allele is associated with an increase in global *CDD* gene expression. We also found a consistent degree of ASE, with the deleted allele expressing 3- to 7-fold higher mRNA levels than the

inserted allele, a result consistent with the global gene expression analysis. Because the functional experiments were done in lymphoblastoid cell lines show notable differences in the transcriptional activities of the deleted and C alleles, we infer that this could also happen in patients *in vivo* and therefore that the rs3215400 variant could be the causal variant.

On the basis of our findings, we propose a model in which the absence of an E2F site within the CDD promoter enhances CDD transcription. This could also take place in normal tissues. In particular, cell cytotoxicity could be accentuated by the elevated proliferation rate observed in the skin of the palm and sole, rendering them more sensitive to the cytotoxic effects of the 5-FU (21). This model may explain the capecitabine-related HFS variability found in treated patients.

Although our finding requires replication through more extensive and independent series of patients, our results provide evidence that rs3215400 in the CDD gene is a risk factor for HFS. An independent series of patients treated with capecitabine enrolled in a clinical trial (Standard Versus Continuous Capecitabine in Advanced Breast Cancer, NCT00418028) is being collected for validation.

This pharmacogenetic study provides new insight into the clinical toxicity associated with capecitabine treatment.

Disclosure of Potential Conflicts of Interest

M. Martín has received speaker's honoraria from Roche.

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Effect of *ABCB1* and *ABCC3* Polymorphisms on Osteosarcoma Survival after Chemotherapy: A Pharmacogenetic Study

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Abstract

Background: Standard treatment for osteosarcoma patients consists of a combination of cisplatin, adriamycin, and methotrexate before surgical resection of the primary tumour, followed by postoperative chemotherapy including vincristine and cyclophosphamide. Unfortunately, many patients still relapse or suffer adverse events. We examined whether common germline polymorphisms in chemotherapeutic transporter and metabolic pathway genes of the drugs used in standard osteosarcoma treatment may predict treatment response.

Methodology/Principal Findings: In this study we screened 102 osteosarcoma patients for 346 Single Nucleotide Polymorphisms (SNPs) and 2 Copy Number Variants (CNVs) in 24 genes involved in the metabolism or transport of cisplatin, adriamycin, methotrexate, vincristine, and cyclophosphamide. We studied the association of the genotypes with tumour response and overall survival. We found that four SNPs in two ATP-binding cassette genes were significantly associated with overall survival: rs4148416 in *ABCC3* (per-allele HR = 8.14, 95%CI = 2.73-20.2, *p-value* = 5.1×10^{-5}), and three SNPs in *ABCB1*, rs4148737 (per-allele HR = 3.66, 95%CI = 1.85–6.11, *p-value* = 6.9×10^{-5}), rs1128503 and rs10276036 ($r^2 = 1$, per-allele HR = 0.24, 95%CI = 0.11–0.47 *p-value* = 7.9×10^{-5}). Associations with these SNPs remained statistically significant after correction for multiple testing (all corrected *p-values* [permutation test] ≤0.03).

Conclusions: Our findings suggest that these polymorphisms may affect osteosarcoma treatment efficacy. If these associations are independently validated, these variants could be used as genetic predictors of clinical outcome in the treatment of osteosarcoma, helping in the design of individualized therapy.

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Introduction

Osteosarcoma is the most frequent malignant bone tumour in children and adolescents. Standard treatment of osteosarcoma is based on a combination of different drugs: neoadjuvant therapy with methotrexate, cisplatin, and adriamycin followed by surgery and post-operative chemotherapy (methotrexate, cisplatin, adriamycin, cyclophosphamide, and vincristine). Despite this, approximately 30% of patients relapse or develop metastasis [1].

Clinical response to chemotherapeutics is a complex trait that is influenced by genetic and environmental factors. Anticancer therapies have a narrow therapeutic range so that a higher concentration in the patient's body causes toxicity and a lower concentration reduces the efficacy of the drug. Interindividual differences in pharmacokinetics and pharmacodynamics determine the global response and toxicity profile of each drug. In this process, the genes involved are the ones that control drug absorption, distribution, metabolism, and excretion. The majority of metabolism reactions are catalyzed by the cytochrome P450 (CYP) enzymes [2]. Many chemotherapeutic agents are also metabolized by glutathione S-transferases (GSTs), phase II detoxification enzymes that catalyse the conjugation of glutathione (GSH) to a wide variety of xenobiotics [3]. Two types of transport superfamilies, ATP-binding cassette (ABC proteins) [4] efflux pumps and solute-linked carrier (SLC) influx proteins [5] are responsible for the majority of drug transport [6]. Most of the drug metabolizers and transporters contain many genetic polymorphisms, which might cause large interindividual variability in the plasma concentration of drugs.

Pharmacogenetic studies have shown that germline polymorphisms in genes related to drug metabolism and transport can have a major effect on the pharmacokinetics and pharmacodynamics of these drugs [6]. We previously performed a study of the nucleotide excision DNA repair pathway in relation to response to cisplatin and observed an association between osteosarcoma outcome and a polymorphism in *ERCC2* gene [7]. Nevertheless, a large portion of the interindividual variability in therapeutic response remains unexplained.

Since mechanisms of transport and metabolism are shared between drugs, genetic variation could affect the bioavailability of more than one drug when they are used in combination, thus affecting the global response to treatment or causing adverse drug events. To address this, pharmacogenetic studies will need to focus on integration of multiple drug pathways to allow a more complete analysis of genetic factors influencing drug efficacy and toxicity.

In the current study, we studied a comprehensive set of SNPs and CNVs that characterize the genetic variation of the multiple metabolic and transport pathways of drugs used in osteosarcoma treatment and their association with drug response.

Methods

Patients, treatments, and clinical variables

One hundred and two consecutive patients diagnosed with osteosarcoma at the University Clinic of Navarra, Pamplona, Spain, between 1986 and 2009 were enrolled in this study. All samples were obtained with written informed consent from patients, their parents, or both. Ethical approval of the study was granted by the Ethics Committee of the University Clinic.

Patients were treated preoperatively with intravenous (i.v.) adriamycin (3 courses at 25–30 mg/m^2/day for 3 days), i.v. methotrexate (4 courses of up to 14 g/m^2/day for 1 day) and intra-arterial cisplatin (3 courses at 35 mg/m^2/day for 3 days). After surgery, the adjuvant chemotherapy included methotrexate (10 g/m^2/day for 1 day and folinic acid rescue) and alternate cycles of i.v. cisplatin/adriamycin or i.v. actinomycin D (0.45 mg/m^2/day for 3 days), cyclophosphamide (500 mg/m^2/day for 3 days), and vincristine (1.5 mg/m^2/day for 1 day) for up to 48 weeks of treatment.

Response to treatment was determined histologically as the percentage of necrosis induced in the tumour after neoadjuvant chemotherapy. Patients with less than 90% necrosis were classified as poor responders and those with 90% necrosis or more, as good responders [8]. Overall survival was considered as the time from tumour diagnosis to death. Event-free-survival (EFS) was considered as the time from tumour diagnosis to the first of disease recurrence, development of lung or bone metastases, and/or death. Patients who were alive at the last follow-up evaluation (January, 2010) were censored at that time. Other clinical data including age, sex, tumour location, metastatic events (both at diagnosis and during follow-up) and relapses (disease recurrence in the same bone) were systematically recorded from the clinical records. Only conventional high-grade osteosarcomas were included, regardless of metastatic stage at diagnosis.

DNA extraction and quantification

Genomic DNA was extracted from peripheral blood lymphocytes using standard phenol-chloroform extraction protocols. DNA was quantified using PicoGreen (Invitrogen Corp., Carlsbad, CA).

Candidate genes and selection of polymorphisms

A total of 24 candidate genes reported to be involved in the metabolism or influx/efflux of the five drugs (cisplatin, adriamycin, methotrexate, vincristine, and cyclophosphamide) were selected, based on the information available in the Pharmacogenomics Knowledge database PharmaGKB (www.pharmgkb.com). These genes encode the following proteins:

Transporters: ABCA3, ABCB1, ABCC1, ABCC2, ABCC3, ABCC4, ABCG2, ABCC6, SLC31A1, SLCO6A1, SLC19A1;

Phase I metabolism enzymes: MPO, SOD1, ALDH1A1, CYP3A4, 3A5, 2A6, 2B6, 2C8, 2C19, 2C9;

Phase II metabolism enzymes: GSTM1, GSTP1 and GSTT1.

SNPs were selected across all these genes except for the *GSTT1* gene. CNVs were studied in the *GSTT1* and *GSTM1* genes.

TagSNPs for the selected genes were defined using Haploview software v.4.0 (http://www.broad.mit.edu/mpg/haploview) with an r^2 threshold of 0.8 and a minimum minor allele frequency of 0.05. All defined tagSNPs were selected for possible genotyping except those in the *ABCC4* gene, for which an excessively elevated number of tagSNPs was defined and so a subset of 57 of these were selected.

In addition, both SNPs with potentially functional effects (causing amino acid changes, potentially causing alternative splicing, in the promoter region, in putative transcription factor binding sites, or disrupting miRNAs and their targets) identified using the bioinformatics tool PupaSuite (http://bioinfo.cipf.es/pupasuite/www/index.jsp), and other functional SNPs already described in the literature were selected.

This preliminary list of SNPs was filtered using as criteria suitability for the Illumina genotyping platform (selecting only those with an assay score >0.6, associated with a high success rate) and minor allele frequencies (MAFs) of at least 5%.

A final number of 366 SNPs relevant to this study was included in an oligonucleotide pool assay for analysis using the Illumina Veracode technology (Illumina Inc., San Diego, CA).

SNP Genotyping

300 ng of DNA for each sample were genotyped using the GoldenGate Genotyping Assay with Veracode technology according to the published Illumina protocol. Data were analysed with GenomeStudio software for genotype clustering and calling. We excluded from further analysis SNPs with a call rate <0.95 and SNPs that deviated from Hardy-Weinberg equilibrium.

Duplicate samples and CEPH trios (Coriell Cell Repository, Camden, NJ) were genotyped across the plates. SNPs showing Mendelian allele-transmission errors or showing discordant genotypes were excluded from the analysis.

GSTM1 and *GSTT1* copy number assays

GSTT1 and *GSTM1* copy number was calculated using Taqman Copy Number Assays (Hs00010004_cn probe for *GSTT1* and Hs02575461_cn for *GSTM1*, Applied Biosystems, Foster City, CA) following the manufacturer's protocol on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). *RNAse P* was used as the reference in TaqMan Copy Number Reference Assays. Data were analyzed using absolute quantification of resulting Ct values generated on the sequence detection system. Copy number was estimated using the CopyCaller 1.0 software (Applied Biosystems). Each sample was evaluated in triplicate.

Statistical analysis

Associations between tumour response and genotypes was assessed using logistic regression analysis [9], comparing genotype frequencies in poor responders versus good responders and estimating odds ratios (OR). Homozygotes for the most frequent allele were used as the reference group. SNPs were also assessed in relation to overall survival and event free survival (EFS) using Cox

regression analysis [10]. Metastasis at diagnosis (no, yes) was included as covariate in multivariable logistic regression and Cox regression analyses.

A permutation test was used to estimate *p-values* corrected for multiple testing. Each replicate consisted of randomly assigning the set of three variables, response to treatment, survival status and analysis time, across subjects and then carrying out the association analyses for each of the 348 successfully genotyped variants and each of the two outcomes (response to treatment and survival). The minimum *p-value* of these 696 (=348×2) tests was then recorded. Ten thousand such replicates were carried out and the corrected *p-values* were estimated as the proportion of replicate *p-values* less than the corresponding unadjusted *p-value*. Reported *p-values* are uncorrected for multiple testing, unless otherwise stated. Only SNP associations with corrected *p-values* <0.05 were considered statistically significant.

Haplotypes containing rs1045642, rs2032582 and rs1128503 in *ABCB1* were inferred by PHASE software, version 2.0. Association with overall survival was then assessed using Cox-regression analysis. Kaplan-Meier curves were generated using SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). All other analyses were carried out using PLINK or R (version 2.6.0.2).

Results

Patients

The main clinical data for the 102 osteosarcoma patients are presented in Table 1. The median age at diagnosis was 14 years (range 3 to 34 years). At the time of diagnosis, 21% of the patients already presented metastasis, while 22% developed metastasis during follow-up. The median follow-up time was 231 months (range 3–303).

Response to treatment (necrosis) data were available for 91 patients and overall survival data were available for 101 patients. The percentage of good responders to therapy was 54 and the median overall survival was 219 months.

Of the clinical variables analyzed, metastasis at diagnosis was found to be associated with increased risk of death (HR = 2.92, 95%CI = 1.35–6.28, *p-value* = 0.006).

Association between polymorphisms and clinical data

A total of 346 SNPs out of 366 and two CNVs were successfully analyzed. Eleven patients were removed for low genotyping call rate (<95%), so finally 91 patients were successfully analyzed.

Results from the analysis of overall survival are presented in Table 1 and Figure 1. We identified four SNPs (two of them in complete linkage disequilibrium) in two genes that were associated with overall survival. The T allele of the synonymous SNP rs4148416 (G1013G) in the *ABCC3* gene was associated with higher risk of death (per-allele HR = 8.14, 95%CI = 2.73–20.2, *p-value* = 5.1×10^{-5}, Figure 1A). In particular, the estimated five-year survival rate for patients carrying the CC genotype of rs4148416 was 78% compared to 20% for heterozygous patients.

The G allele of rs4148737, an intronic SNP located in the *ABCB1* gene, was associated with poorer overall survival (per-allele HR = 3.66, 95%CI = 1.85–6.11, *p-value* = 6.9×10^{-5},) Figure 1B). The estimated five-year survival rate for patients carrying the common AA genotype was 93% compared to 38% for patients homozygous for the G allele. The minor alleles of two other SNPs (rs1128503 and rs10276036) in this gene, in complete linkage disequilibrium (LD, $r^2 = 1.0$), and in partial LD with rs4148737 ($r^2 = 0.48$), were also associated with better overall survival (per-allele HR = 0.24, 95%CI = 0.11–0.47, *p-value* = 7.9×10^{-5}, Figure 1C). For these two SNPs, the estimated five-year survival rate for common homozygotes was 49% compared to 100% for patients homozygous for the rare allele. These results did not change substantially after adjusting for metastasis at diagnosis (Table 2). All three associations remained statistically significant after correction for multiple testing (corrected *p-value*≤0.030).

Table 1. Clinical characteristics of osteosarcoma patients (*N* = 102).

	Patients	
	N	%
Age at diagnosis (years)		
Median	14.8	
Range	3–34	
Sex		
Female	45	44.1
Male	57	55.9
Location		
Femur	51	50.0
Tibia	38	37.3
Arm	7	6.9
Central	6	5.9
Response to treatment		
Good	52	57.1
Poor	39	42.9
Metastasis		
No	59	57.8
At diagnosis	21	20.6
At follow up	22	21.6
Status		
Alive	72	71.3
Dead	29	28.7
Relapse		
No	85	83.3
Yes	17	16.7

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We also studied these SNPs in relation to event-free survival (EFS) and highly consistent results were observed (Table 2). None of the SNPs analyzed were significantly associated with tumour response after correction for multiple testing.

A combination of three SNPs located in *ABCB1* (rs1045642 / rs2032582/ rs1128503) was previously described as putatively functional in several studies [4],[11],[12]. We explored whether there is a haplotype formed by these three SNPs that is more significantly associated with survival in these patients. We observed two frequent haplotypes, one formed by the combination of the three common alleles (CGC) with a frequency of 0.47 and the other comprising the three rare alleles (TTT) with a frequency of 0.39. Considering CGC as reference, the TTT haplotype was associated with better survival (HR = 0.31, 95%CI = 0.15–0.62, *p-value* = 0.001). The other haplotypes observed had lower frequency and were not statistically significantly associated with survival. Nevertheless, the estimated HR for the two haplotypes containing the rare T allele in rs1128503 (CGT and CTT) were consistent with it having a protective effect (HR = 0.38 and 9.78×10^{-6}, respectively) in contrast with the other two haplotypes containing the C wild-type allele (TGC and TTC; HR = 1.26 and 1.95,

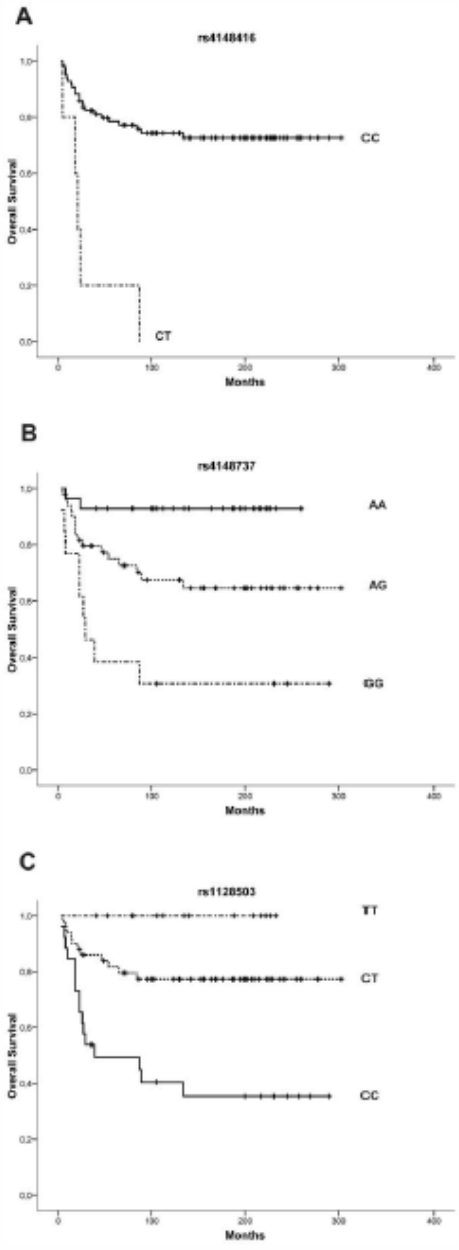

Figure 1. Kaplan-Meier survival curves for osteosarcoma patients according to genotype for (A) rs4148416 in *ABCC3* ($X^2=21.4$, $p\text{-value}=3.8\times10^{-6}$); (B) rs4148737 in *ABCB1* ($X^2=18.4$, $p\text{-value}=1.0\times10^{-4}$); and (C) rs1128503 or rs10276036 in *ABCB1* ($X^2=20.9$, $p\text{-value}=2.9\times10^{-5}$).
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respectively). These results suggested that the haplotypes were no more informative than the rs1128503 SNP alone.

Regarding the CNVs analyzed, genotype data for *GSTM1* and *GSTT1* were available for 98 and 99 patients, respectively. The frequency of the homozygous gene deletion was 52% (51 patients) for *GSTM1* and 19% (19 patients) for *GSTT1*. There was no evidence that either of these two polymorphisms were associated with any of the clinical outcomes considered.

Discussion

This study assessed 346 SNPs and 2 CNVs in 24 key genes involved in platinum, adriamycin, methotrexate, vincristine, and cyclophosphamide pathways, and is the most comprehensive pharmacogenetic study in osteosarcoma patients to date.

The use of large scale genotyping methods to screen multiple drug pathways has allowed us to identify four SNPs (two of them in total LD) that are highly associated with overall survival and that might be useful prognostic markers in these patients.

To our knowledge, the majority of the pharmacogenetic studies are biased towards candidate polymorphisms in pharmacogenetic genes already described as functional in previously published data. However, given the complexity and the lack of understanding of both genetic variation effects and regulation of chemotherapy action, these polymorphisms explain only a portion of the observed phenotypic variability in the drug outcome. In our study, we have assessed all genes well-known to be involved in the metabolism and transport pathways of the drugs used in osteosarcoma treatment. In order to avoid any bias, we selected not only already described and potentially functional polymorphisms, but also tagSNPs, to obtain a more comprehensive view and to detect novel markers that could play a role in the interindividual differences observed of outcome risk in osteosarcoma patients.

ABCC3 is a member of the multidrug resistance protein (MRP) family and is expressed in liver, gallbladder, kidney, and gut [13],[14]. Main substrates of *ABCC3* are bile salts [15], but it also transports anticancer drugs, among which methotrexate [16], [17]. Vincristine, doxorubicin, and cisplatin have also been suggested to be *ABCC3* substrates, but there are no clear results [17], [18]. The expression of *ABCC3* mRNA has been related to drug resistance [16] but to date only limited studies have been published studying polymorphisms in *ABCC3*. Only promoter SNPs and non-synonymous SNPs have been investigated as potentially functional variants [19,20], but, to our knowledge, there are no studies showing association between these genetic *ABCC3* variants and survival after treatment in cancer patients. In the present study we found that SNP rs4148416 in this gene was associated with an 8-fold risk of low survival and this is the first evidence of its clinical relevance. This SNP leads to a synonymous change (G1013G) at exon 22. The other SNPs we found associated with osteosarcoma survival are located in the *ABCB1* gene. This gene is well-known and encodes a P-glycoprotein, an ATP-driven efflux pump, that is overexpressed in many tumours and confers multidrug resistance [21]. Of the drugs administered to these patients, both doxorubicin and vincristine are transported by this pump [22]. There are three variants in LD that have been studied in detail: 2677G>T/A (rs2032582), 3435C>T (rs1045642) and

Table 2. Genes and polymorphisms associated with overall survival (OS) and event free survival (EFS).

SNP	Genotype	N	5-year survival rate	OS HR* (95%CI) P-value	OS Adjusted**HR (95%CI) P-value	EFS HR (95%CI) P-value
ABCC3						
rs4148416	CC	85	78%			
	CT	5	20%			
	per allele T			8.14	7.25	6.33
				(2.73–20.2)	(2.62–20.1)	(1.79–12.7)
				0.000051	0.00014	0.00028
ABCB1						
rs4148737	AA	28	93%			
	AG	49	75%			
	GG	13	38%			
	per allele G			3.66	2.83	2.60
				(1.85–6.11)	(1.56–5.12)	(1.24–3.22)
				0.000069	0.00061	0.00051
rs1128503	CC	26	49%			
	CT	50	82%			
	TT	14	100%			
	per allele T			0.24	0.27	0.42
				(0.11–0.47)	(0.13–0.54)	(0.29–0.81)
				0.000079	0.00023	0.0021
rs10276036	TT	26	49%			
	TC	50	82%			
	CC	14	100%			
	per allele C			0.24	0.27	0.42
				(0.11–0.47)	(0.13–0.54)	(0.29–0.81)
				0.000079	0.00023	0.0021

*HR: Hazard Ratio.
**Analysis adjusted for metastasis at diagnosis.
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1236C>T (rs1128503). The first is a non-synonymous change, while the other two are synonymous. These three SNPs have been studied both individually and as a haplotype, but the results have been inconsistent [4] [23] [11] [24]. The rare allele of rs1045642 has been reported to be associated with reduced P-glycoprotein activity, both alone and in combination with the rare alleles of rs2032582 and rs1128503 [23], [25]. These latter two variants have also been reported to have independent functional effects in different studies [12], [25], [26], [27]. However, it is still not clear which is/are the functional variant/s in this gene.

In our study, of these three variants, only 1236T>C (rs1128503) was strongly associated with survival in our series of patients. This SNP leads to a synonymous change at residue 412 of the protein and is well-known but there is no clear consensus on its functional significance [28]. Some studies found increased response in the presence of the T allele [29], [30] while others found the opposite [31], or no genetic effect [32]. In our study, the T allele was associated with increased survival. To see if the association found was due to this single variant or to a combination of the three already described polymorphisms we also analyzed the effect of the haplotypes on overall survival. The results were consistent with a single main effect for rs1128503. Consistent results were reported by Balcerczak and colleagues in colorectal cancer patients [12].

We found another SNP, rs10276036, associated with survival that was in complete LD with the previous one. It is located in intron 9 and has been linked with reduced area under the curve (AUC) of SN-38, the active metabolite of CPT-11, [33]; however its functional significance is unknown. The effect observed could be explained by its correlation with rs1128503, rather than by rs10276036 itself. Apart from the already described SNPs, we found a polymorphism located in intron 17 (rs4148737) also strongly associated with survival. This SNP was in low LD with the other two SNPs. Further functional studies are needed to elucidate which is the causal variant in the *ABCB1* gene.

We hypothesize that the variants associated with overall survival could have an effect on the efflux of the drugs used in the treatment of osteosarcoma, thus impairing the response to the treatment and therefore the overall survival. Although we did not find a statistical association between these variants and tumour response, this could be explained by the fact that tumour response is evaluated after neoadjuvant therapy and at this point patients have been treated exclusively with methotrexate, cisplatin, and adriamycin. Therefore, the tumour necrosis data does not evaluate

the effect of the vincristine and cyclophosphamide drugs used after surgery. Since ABCB1 is known to transport vincristine, ABCC3 could also be involved in this process [18,34], and the transport mechanisms for cyclophosphamide are still unknown, we postulate that genetic variation in these transporters could play a role in the effectiveness of the whole treatment and influence the overall survival.

In conclusion, this study identified four significant SNPs in two drug transporter genes associated with overall survival in osteosarcoma patients. After validation in large and well-defined sets of patients to confirm the associations, these variants could be useful as prognostic markers in these patients.

Furthermore, the approach used in this study, integrating multiple drug pathways and studying an increased number of polymorphisms, may be extended to future pharmacogenetic studies to provide a wide scenario of genetic factors influencing drug efficacy and toxicity. The applicability of high-throughput gene chips that allow simultaneous analysis of multiple polymorphisms will facilitate research in this field.

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Author Contributions

Conceived and designed the experiments: DC APG AGN IS JB. Performed the experiments: DC LM MZD. Analyzed the data: DC GP. Contributed reagents/materials/analysis tools: APG APM IC BM LS MZD AGN. Wrote the paper: DC AGN APG.

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APPENDIX II

Other publications

1. Nat Genet. 2011 Jun 19;43(7):663-7.

Exome sequencing identifies MAX mutations as a cause of hereditary pheochromocytoma.

Comino-Méndez I, Gracia-Aznárez FJ, Schiavi F, Landa I, Leandro-García LJ, Letón R, Honrado E, Ramos-Medina R, Caronia D, Pita G, Gómez-Graña A, de Cubas AA, Inglada-Pérez L, Maliszewska A, Taschin E, Bobisse S, Pica G, Loli P, Hernández-Lavado R, Díaz JA, Gómez-Morales M, González-Neira A, Roncador G, Rodríguez-Antona C, Benítez J, Mannelli M, Opocher G, Robledo M, Cascón A.

Hereditary pheochromocytoma (PCC) is often caused by germline mutations in one of nine susceptibility genes described to date, but there are familial cases without mutations in these known genes. We sequenced the exomes of three unrelated individuals with hereditary PCC (cases) and identified mutations in MAX, the MYC associated factor X gene. Absence of MAX protein in the tumours and loss of heterozygosity caused by uniparental disomy supported the involvement of MAX alterations in the disease. A follow-up study of a selected series of 59 cases with PCC identified five additional MAX mutations and suggested an association with malignant outcome and preferential paternal transmission of MAX mutations. The involvement of the MYC-MAX-MXD1 network in the development and progression of neural crest cell tumours is further supported by the lack of functional MAX in rat PCC (PC12) cells and by the amplification of MYCN in neuroblastoma and suggests that loss of MAX function is correlated with metastatic potential.